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Investigation of functional and morphological integrity of freshly isolated and cryopreserved human hepatocytes

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Abstract

There is a pressing need for alternative therapeutic methods effective in the treatment of patients with liver insufficiency. Isolated human hepatocytes may be a viable alternative or adjunct to orthotopic liver transplantation in such patients. The purpose of this study was to evaluate the viability and functional integrity of freshly isolated and cryopreserved human hepatocytes, in preparation for a multi-center human hepatocyte transplantation trial. We are currently processing transplant-grade human parenchymal liver cells from nondiseased human livers that are obtained through a network of organ procurement organizations (OPOs). Thus far, sixteen hepatocyte transplants have been performed using hepatocytes processed by our methods. At the time of referral all specimens were deemed unsuitable for transplantation due to anatomical anomalies, high fat content, medical history, etc. Hepatocytes were isolated from encapsulated liver sections by a modified two-step perfusion technique. Isolated cells were cryopreserved and stored in liquid nitrogen for one to twelve months. The total yield of freshly isolated hepatocytes averaged 3.7×10^7 cells per gram of wet tissue. Based on trypan blue exclusion, fresh preparations contained an average of 85% viable hepatocytes vs. 70% in cryopreserved samples. The plating efficiencies of cells seeded immediately after isolation ranged from 87% to 98%, while those of cryopreserved/thawed cells were markedly lower. Flow cytometry analysis of cells labeled with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) showed that there was no significant difference in viability compared with trypan blue staining. Both freshly isolated hepatocytes and those recovered from cryopreservation showed typical and intact morphology as demonstrated by light and electron microscopy. The product of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reaction was always expressed more intensely in cultures of freshly isolated hepatocytes. Measurements of lactate dehydrogenase (LDH) leakage were inversely correlated with trypan blue exclusion and CFSE labeling. Energy status, evaluated by the intracellular ATP concentration measurements, and various liver-specific functions such as urea synthesis and metabolism of 7-ethoxycoumarin were maintained both in fresh and cryopreserved/thawed hepatocytes. However, the activities were expressed at different levels in thawed cells. These data illustrate the importance and feasibility of human hepatocyte banking. In addition, it is clear that further refinements in the methods of hepatocyte isolation and cryopreservation are needed to utilize more fully these valuable cells in the clinic.

Introduction

Hepatic parenchymal cells (hepatocytes) represent 60–70% of all cells in the liver and perform the majority of liver-related functions including metabolic transformation of xenobiotics. Enzymatic isolation of hepatocytes, first introduced for laboratory animals in the mid

1960s (Howard et al. 1967) and significantly improved in recent years, has enabled rapid increases in the use of human hepatocytes in biomedical research. In recent years, many reports have confirmed that properly isolated and prepared human hepatocytes retain their metabolic activity and cellular morphology, and can provide a valuable *in vitro* liver model (Li et al. 1997,

Hasset et al. 1998). Potential research applications of isolated hepatocytes include the biotransformation of drugs and hormones, the toxicity of drugs and environmental chemicals, the purification and characterization of hepatic gene products, the regulation of liver gene expression, and the study of liver diseases such as cancer and hepatitis C. Potential clinical applications include cell transplantation and artificial liver support to treat a variety of conditions, including hepatic-based inborn errors of metabolism, chronically diseased liver, and acute liver failure. Some recent clinical trials have shown that isolated hepatocytes may be useful for this kind of hepatic support (Strom et al. 1997, Fox et al. 1998).

The use of human hepatocytes, both for basic research and clinical trials, has been hampered by the limited availability of adequate numbers of fresh, viable cells due to the ongoing shortage of liver donors. In 1996, for example, 4058 livers were transplanted while 8221 patients were on the UNOS liver transplant waiting list in early 1997 (Bode 1997). Thus, there is no surplus of healthy organs from which freshly isolated hepatocytes can be prepared when needed.

A practical solution to this problem is cryopreservation and banking of hepatocytes. In most cases, the yield of primary hepatocytes from a single lobe of a fresh human liver is greater than 5×10^9 cells, sufficient for a hepatocyte transplant but well in excess of a large-scale *in vitro* assays. Although cryopreservation of isolated hepatocytes has been one of the most challenging steps in human hepatocyte banking, it has been reported in some studies that the freeze-thaw cycle may result in the loss of cell viability and the deterioration of culturing efficiency (Coundouris et al. 1993). Moreover, the fine structure and metabolic functions of human hepatocytes may be significantly altered after freezing and thawing process (Powis et al. 1989). In contrast, it has been reported that cryopreserved hepatocytes transplanted intrasplenically or into the portal vein have a beneficial effect (Maganto 1990, Strom et al. 1997, 1999). Thus, there is a continuing need for further improvement in the methods of long-term cryopreservation and recovery of human hepatocytes to maximize the efficient use and minimize the waste of this valuable resource.

In this study, we compared the *in vitro* functional and morphological integrity of freshly isolated human hepatocytes and those recovered from cryopreservation. Our results show that cryopreserved/thawed hepatocytes stored in liquid nitrogen for extended periods retained their morphological integrity and expressed

functional activities at levels close to the freshly isolated primary cells.

Materials and methods

Chemicals

Media used for the hepatocyte isolation, culture and cryopreservation including Hanks' and Earle's Balanced Salt Solution (HBSS, EBSS), Leibovitz (L-15) and Waymouth's 752/1, all supplements, dimethyl sulfoxide (DMSO), lactate dehydrogenase (LDH) determination kit, reagent system for urea nitrogen and periodic acid Schiff (PAS) kit were purchased from Sigma Chemical Corp. (St. Louis, MO) or from GIBCO BRL (Grand Island, NY). Fetal Bovine Serum (FBS) was from Hyclone Laboratories Inc. (Logan, UT). Collagenase was obtained from Boehringer Mannheim Corp. (Indianapolis, IN) (Type P) or Worthington Biochemical Corp. (Freehold, NJ) (Type 1). Percoll was purchased from Pharmacia Biotech. Inc. (Newark, NJ). Fluorescent dye CFSE was from Molecular Probes, Inc. (Eugene, OR). Antibodies (CD45) were from PharminGen (San Diego, CA). University of Wisconsin (UW) solution was obtained from DuPont Pharmaceuticals (Wilmington, DE). Matrigel was from Becton Dickinson (Bedford, MA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was from Aldrich Chem. Co. (Milwaukee, WI). All other reagents and chemicals were of the highest quality obtained from commercial suppliers.

Liver procurement

Human livers were obtained through a network consisting of organ procurement organizations (OPOs). Donor demographic data are presented in Table 1. At the time of referral, all specimens had been deemed unsuitable for transplantation for one or more reasons (e.g., anatomical anomalies, biopsy results, patient medical/social history). All livers were collected from brain-dead donors with informed consent from the next-of-kin. The donated livers were protected from ischemia injury by flushing with ice-cold UW solution immediately after vascular clamping. The interval from initial flush to hepatocyte isolation was never more than 30 h and was often much less. All liver donors were tested negative for known viruses, and all processed livers were handled as transplantable tissue according to FDA (Food and Drug Administration), AATB

Freshly isolated human hepatocytes

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Table 1. Donor demographic data and the total yield of freshly isolated hepatocytes.

Liver #	Sex	Age (years)	Yield (10^7 cells/g tissue)
1	M	36	5.3
2	F	42	5.9
3	M	2.5	3.8
4	M	18	3.1
5	M	65	3.6
6	M	25	2.5
7	M	69	2.8
8	F	4	2.4

(American Association of Tissue Banks) and/or PRTB (Pennsylvania Regional Tissue Bank) standards.

Hepatocyte preparation

All hepatocyte manipulations were performed under sterile conditions in a biosafety cabinet. Hepatocytes were isolated from an encapsulated liver sample (preferably the left lateral segment) by a modified two-step perfusion technique introduced by Seglen (1976). Briefly, the dissected lobe was placed in the custom-made perfusion apparatus and two to five hepatic vessels were cannulated with tubing attached to a multi-channel manifold. A liver fragment (150–500 g) was perfused initially (recirculation technique) with calcium free HBSS supplemented with 0.5 mM EGTA (ethylene glycol-bis [β -aminoethyl ether]-N,N,N',N'-tetraacetic acid) for 20–30 min and then with 0.05% collagenase dissolved in L-15 medium at 37 °C until the tissue was fully digested. The digested liver was removed, immediately cooled with ice-cold L-15 medium and the cell suspension was strained through serial progressively smaller stainless steel sieves, with a final filtration through a 100 μ m and 60 μ m nylon mesh. The filtered cell suspension was aliquoted into 250 ml tubes and centrifuged three times at 40 \times g for 3 min at 4 °C. After the last centrifugation the cells were resuspended, combined in one tube and placed on ice. In three out of eight cases (in which the initial viability was lower than 80%), the crude hepatocyte suspension (washed and filtered only) was purified on a Percoll gradient. The optimal concentration of Percoll (determined by titration) varied from one preparation to another due to the differences in cellular fat content. Cells were centrifuged at 65 \times g for 12 min at 4 °C. The supernatant, containing damaged or dead hepatocytes, nonparenchymal cells and debris,

was removed and pelleted hepatocytes were washed twice (at 40 \times g for 3 min) in L-15 medium. The purity of Percoll-purified cells was verified by CD45 staining of 10^5 cells in a Cytospin preparation (Cytospin 2, Shandon Inc., Pittsburgh, PA).

Hepatocyte culture

The final cell suspension was centrifuged, and the medium aspirated. The isolated hepatocytes were resuspended (7.5×10^5 /ml) in the plating medium (combination of EBSS and Waymouth's 752/1) supplemented with: 10% FBS, 100 ng/ml insulin, and 0.1 μ M dexamethasone, dispensed into 6-well (1.5×10^6 cells/well), or 96-well (7.5×10^4 cells/well) plates precoated with type I collagen and incubated at 37 °C in 5% CO₂/95% air. Once hepatocyte attachment was completed, the plating medium was removed and cell monolayer was covered with serum-free culture medium (combination of EBSS and Waymouth's 752/1 media supplemented with: 100 ng/ml insulin, 1 μ g/ml glucagon, 10 μ units/ml somatotropin, 1 μ M thyroxine, 60 μ g/ml proline, 75 μ g/L hydrocortisone, 6 μ g/ml linoleic acid, 3 nM of selenium, and 0.1 μ M dexamethasone), or sandwiched with Metrigel overlay. Cultures were fed every 24 h by complete medium exchange.

Hepatocyte cryopreservation and thawing

The cells were sedimented and resuspended at a density of 7–10 $\times 10^6$ /ml of UW solution and 1 ml aliquots of hepatocyte suspension were transferred to 2 ml cryogenic Corning vials. An equal volume of cryoprotectant mixture containing 40% UW solution (vol/vol), 20% DMSO (vol/vol), and 40% FBS (vol/vol) was slowly (in 2 min) added to the cells. A computerized controlled-rate freezing apparatus (Cryomed, Forma Scientific, Marietta, OH) was used to cryopreserve the cells. The freezing rate was –3 °C/min down to –4 °C and –10 °C/min thereafter. Frozen cells were quickly transferred to the liquid nitrogen vapor phase and kept for 1–12 months. For cell thawing the vials were removed from LN₂ and placed in 37 °C water bath. The cells were gently pipetted into 15 ml centrifuge tubes, slowly resuspended in ice-cold L-15 medium and centrifuged twice at 40 \times g for 3 min. After centrifugation the cells were resuspended in UW solution and incubated at 37 °C for 30 min before plating. Only two preparations of hepatocytes (liver # 3 and # 8) did not require Percoll purification after thawing.

Determination of hepatocyte viability and plasma membrane integrity

The yield and viability of freshly isolated or thawed cells were determined by mixing 50 μ l hepatocyte suspension with the same volume of trypan blue solution and counting stained (non-viable) and unstained (viable) cells in a hemocytometer. Preparations with greater than 80% viability were used for other applications without further purification.

For rapid assessment of hepatocyte viability, the cells were labeled with fluorochromes. Three million hepatocytes were suspended in L-15 medium supplemented with 5% FBS and fluorescein dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) was added from a stock solution to a final concentration of 5 μ M. The cells were incubated for 15 min at 37 °C, and centrifuged twice in L-15 medium. Fluorescence of labeled cells was verified by flow cytometry analysis according to standard procedures using an Elite Epics sorter (Coulter Corp., Miami, FL).

Plating efficiency was determined by calculating the percentage of attached cells after 12 h incubation at 37 °C. Viability of fresh and cryopreserved/thawed hepatocytes cultured for 12 h in 96-well plates was evaluated by using the MTT assay. Reduction of a yellow salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), by mitochondrial dehydrogenases in viable cells to a purple formazan precipitate was determined by measuring the optical density (OD) at 570 nm on a plate reader (Microplate, Bio-Tek Instruments, Highland Park, VT).

LDH (lactate dehydrogenase) leakage from freshly isolated or thawed hepatocytes (incubated in suspensions in triplicate for each time point 30 min, 1 and 3 h) was estimated by using the diagnostic kit for kinetic determination of LDH based on the spectrophotometric method of Wroblewski and LaDue (1955). The absorbance was measured at 340 nm using BioSpec-1601 spectrophotometer (Shimadzu Corp., Columbia, MD). LDH leakage was expressed as a percentage of total LDH present in the incubated hepatocyte suspensions, according to Berry et al. (1991).

Hepatocyte function assays

To measure ureagenesis, 1×10^6 cells were suspended in 1 ml of serum-free L-15 medium and incubated with addition of 5 mmol/L ammonium chloride for

0, 1, 2, and 4 h. After incubation the cells were centrifuged and the urea concentration in the medium was measured using a spectrophotometric urea nitrogen test kit.

Energy status of hepatocytes was evaluated by measurement of intracellular ATP content according to the procedure developed by FireZyme Diagnostic Technologies Ltd. Firefly luciferin/luciferase was added to 1×10^5 cells/100 μ l of L-15 medium and the light output assay was measured in triplicate using the Luminometer Model SVT 2.5 (FireZyme Limited, San Diego, CA).

To determine cytochrome P450-dependent metabolic function, 7-ethoxycoumarin (7-EC) metabolism activity was analyzed in hepatocyte incubation medium samples. This assay involved the measurement of 7-hydroxycoumarin (7-HC), 7-hydroxycoumarin-glucuronide (7-HC Gluc.), and 7-hydroxycoumarin-sulphate (7-HC Sulf.) formation after 2 h incubation of viable 1×10^6 cells/ml suspended in Krebs buffer with addition of 25 μ M of 7-EC. Metabolite production was identified by reverse phase HPLC (Waters, Millipore Corp., Milford, MA) as described elsewhere. Briefly, the incubation supernatant was removed, centrifuged at 12000 rpm and then injected (100 μ l) to the chromatographic system. Absorbance was monitored at 230 nm. All standards and reaction conditions were optimized internally.

Microscopy

Culture plates were viewed by phase-contrast microscopy. Fluorescence of CD45-stained or CFSE-labeled cells was inspected by a fluorescent microscope. For morphological and histochemical analysis, 10^5 unfixed hepatocytes were suspended in HBSS with 1% FBS, spun for 5 min in Shandon Cytospin and then stained with hematoxylin and eosin (H&E) or by periodic acid-Schiff (PAS). For electron microscopy, suspensions of hepatocytes were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate then pelleted and embedded in Spurr's resin. Thin sections were stained with lead nitrate and uranyl acetate and examined by a CM12 Philips transmission electron microscopy.

Statistics

Each analytical assay was carried out in at least three cell preparations. Data are expressed as means \pm SE and analyzed using unpaired, two-tailed Student's

t-test for comparisons between fresh and cryopreserved hepatocytes isolated from the same liver. A *p* value <0.05 was considered significant.

Results

Hepatocyte viability and morphology

The total yield of freshly isolated hepatocytes averaged 3.7×10^7 cells per gram of wet tissue (Table 1). The final cell suspension averaged 85%, 93%, and 70% viable hepatocytes respectively, before Percoll purification (freshly isolated), after Percoll, and in cryopreserved samples. Fluorescence of viable CFSE-labeled cells, another measure of viability, averaged 87% before Percoll, 96% after Percoll purification and 72% in cryopreserved samples, which correlated closely with the viability determinations by trypan blue exclusion (Table 2, Figure 1a). The flow cytometric distribution of CFSE-labeled hepatocytes (data not shown) was consistent with the quantitative measures of cell viability shown in Table 2. Purity assessment of Percoll-purified hepatocytes (livers # 4, 5, 6) revealed a significant decrease in the degree of contamination by nonparenchymal cells compared with crude preparations; mean contamination by CD45+ cells was 0.7% and 8.8%, respectively (Figure 1b). The viability of hepatocytes suspended in culture medium, estimated by trypan blue exclusion and CFSF staining, was inversely correlated with LDH leakage. Over a 3 h incubation period, less than 6% of total LDH in fresh hepatocyte suspension was released from damaged cells, 35% after cryopreservation and thawing, and less than 10% after Percoll purification of cryopreserved/thawed cells (Figure 2).

Viable hepatocytes, either freshly isolated or recovered from cryopreservation, showed typical and intact morphology as demonstrated by light microscopy. In suspensions, most of the cells were binucleated and spherical in shape with some signs of translucent blebbing of plasma membrane. Blebs, the most characteristic abnormal feature of cryopreserved hepatocytes, were mainly observed in the cells isolated from fatty livers. Incubation at 37 °C for 30 min resulted in significant improvement of cell morphology and reversal of blebbing. Routine PAS staining demonstrated the presence of intracellular red granules of glycogen, to a similar degree, in both fresh and cryopreserved hepatocytes. Electron microscopy examination showed that freshly isolated cells displayed their typical ultrastructural integrity with intact central nuclei,

mitochondria and endoplasmic reticulum (Figure 3a). The appearance of cryopreserved hepatocytes was similar to that of normal cells. However, moderate signs of cellular derangement were found in some cells, including slightly swollen mitochondria, large lipid droplets, and development of vesicular forms in endoplasmic reticulum (Figure 3b).

Freshly isolated hepatocytes harvested from seven out of eight livers attached to collagen-coated dishes within 5 h after seeding, and formed confluent cultures by 12 h at which point the cells had spread, flattened, and formed a monolayer across the dish (Figure 4a). The plating efficiency of the cells seeded immediately after isolation ranged from 87% to 98% (Table 3). In contrast, at least 8 h was required for attachment of cryopreserved hepatocytes and the plating efficiency was significantly lower as well (22–72%).

One week after plating, both fresh and cryopreserved/thawed cells cultured on collagen alone were still attached but showed some irregularity in shape and morphology such as elongation of cytoplasmic projections and cytoplasm granularity (Figure 4b). In 14-day-old monolayer cultures, continuous hepatocyte deterioration and detachment was observed. In contrast, both fresh and cryopreserved cells cultured with a Matrigel overlay maintained normal morphology for at least 21 days.

The MTT assay demonstrated that viability of cells recovered from cryopreservation was strictly related to the attachment and plating efficiency. The MTT reaction product was always expressed more intensely in cultures of freshly isolated hepatocytes (Figure 5). However, in two cases (livers # 3 and # 6), cryopreservation had no significant influence on the conversion of MTT to formazan.

Table 2. Viability of freshly isolated and cryopreserved/thawed hepatocytes as determined by trypan blue exclusion and CFSE labeling.

Liver #	Hepatocyte preparations			
	Fresh Trypan Blue	CFSE	Cryopreserved Trypan Blue	CFSE
1	91% ± 1.2	93%	72% ± 2.3*	75%
2	89% ± 2.3	91%	74% ± 2.3*	78%
3	94% ± 1.7	95%	85% ± 2.9	82%
4	74% ± 1.2	76%	68% ± 2.3	71%
5	74% ± 1.6	78%	66% ± 2.3*	63%
6	77% ± 1.2	81%	48% ± 1.2*	53%
7	86% ± 1.2	84%	66% ± 0.6*	69%

(*) *p* < 0.05 vs. fresh hepatocytes.

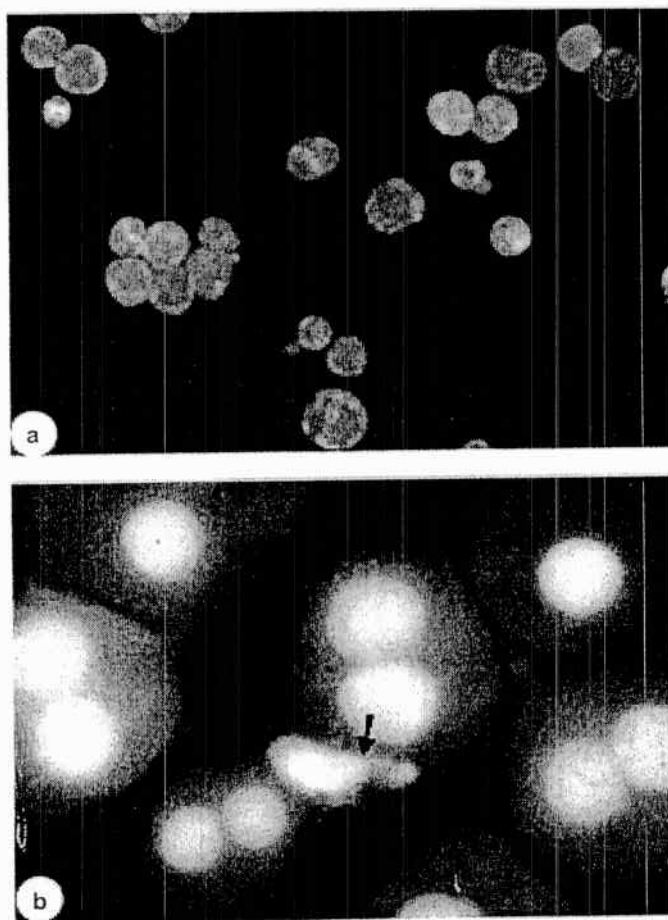


Figure 1. Fluorescent photomicrograph of freshly isolated human hepatocytes. CFSE-labeled viable cells in suspension (a). Percoll-purified hepatocytes verified by CD45 staining in a Cytospin preparation (b); arrow, CD45+ nonparenchymal cell. (a) $\times 200$; (b) $\times 500$.

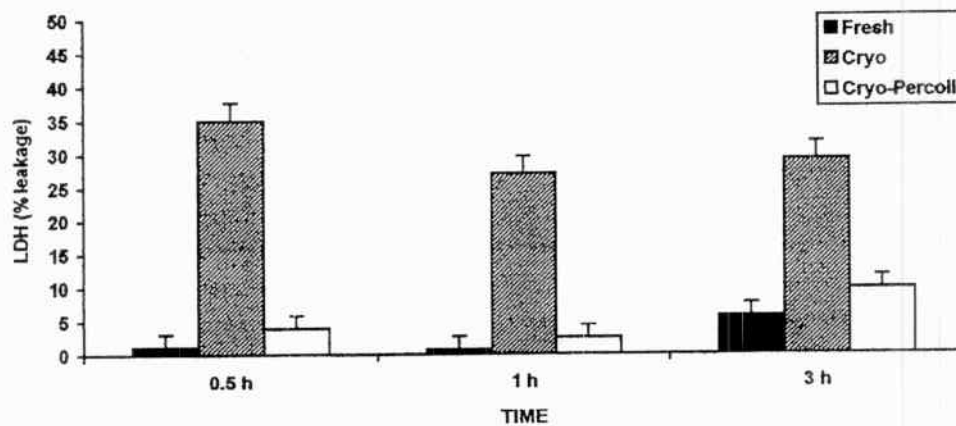


Figure 2. Percent of total LDH leakage from fresh and cryopreserved/thawed hepatocytes into incubation media over time.

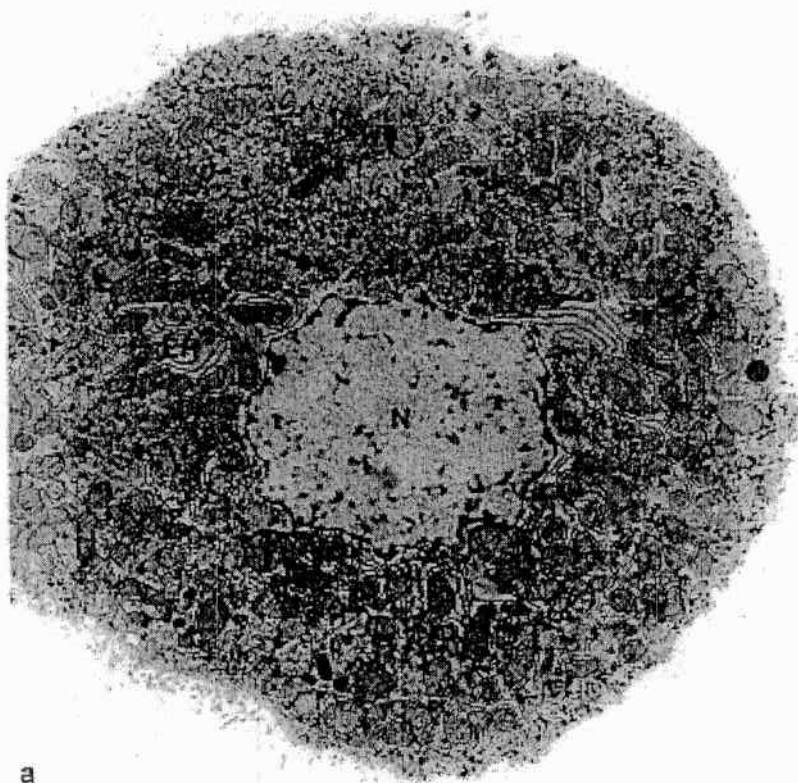


Figure 3.

Hepatocyte function

Table 4 shows values representing the formation of metabolites by freshly isolated and cryopreserved/thawed hepatocytes incubated with 25 μ M 7-EC. Although drug metabolism capacity varied from one liver to another, cryopreserved hepatocytes retained most of the metabolizing activity expressed by freshly isolated cells from the same preparation. The formation of 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide were monitored in all tested samples. In six of eight preparations, 7-HC predominated over 7-HC Gluc. In every case, the initial relative rates of formation of 7-HC and 7-HC Gluc. (i.e., the ratio of 7-HC to 7-HC Gluc.) were maintained through the process of cryopreservation and thawing. In three of eight preparations, 7-HC Sulf. was detected, in each case at a level substantially less than the unconjugated or glucuronidated product. There was a perfect correlation between fresh and

cryopreserved cells in terms of the presence or absence of 7-HC Sulf. The 7-EC metabolizing activities of fresh vs. cryopreserved cells in the various preparations were generally quite similar. In only two out of eight cases was the total metabolite formation significantly lower in cryopreserved cells than in the corresponding freshly isolated cells. The rank order of total activity was identical for the fresh and cryopreserved cells.

Both freshly isolated and those recovered from cryopreservation hepatocytes synthesized and secreted urea into the medium during the 4 h incubation period. No significant difference in urea synthesis was observed between the two groups of cells in the seven studied livers. For fresh cells, the average rate of urea synthesis was 580 nmol/ 10^6 cells/4 h vs. 539 nmol/ 10^6 cells/4 h for cryopreserved hepatocytes. Basal rates of urea synthesis for both hepatocyte preparations obtained from all processed livers are presented graphically in Figure 6.

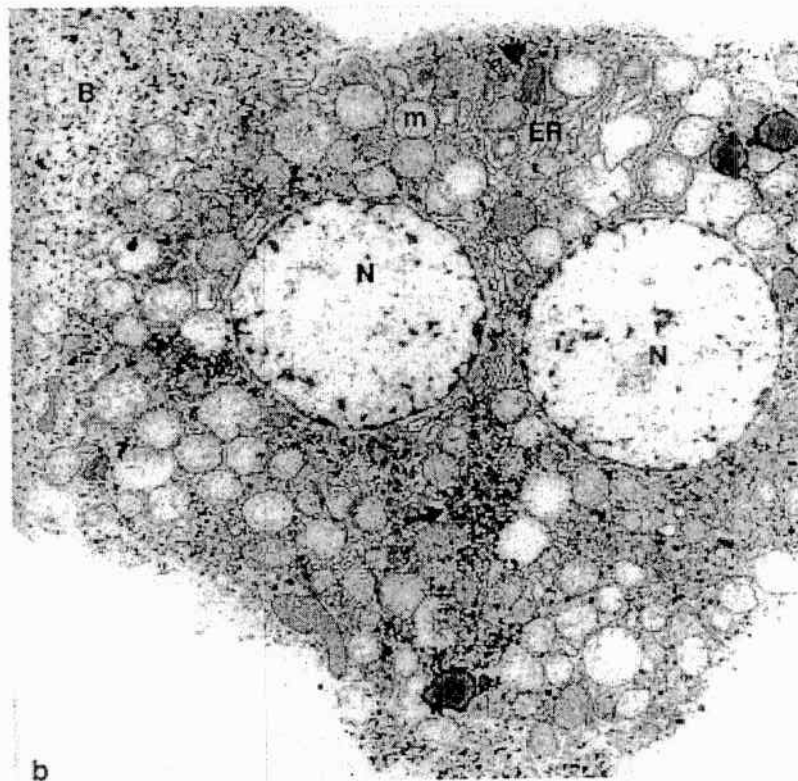


Figure 3. Electron micrographs of freshly isolated (a) and cryopreserved/thawed (b) human hepatocytes. M, mitochondria; N, nucleus; L, lipid droplets; ER, endoplasmic reticulum; arrow, granules of glycogen; B, blebbing of plasma membrane. (a, b) $\times 3000$.

Measurements of the intracellular ATP concentration showed a very consistent pattern of the energy status in both fresh and cryopreserved hepatocytes. Figure 7 shows data from eight liver preparations. The average ATP content of freshly isolated hepatocytes ranged from 8.5 to 12.2 nmol/ 10^6 cells, vs. 6.1 nmol/ 10^6 cells for cryopreserved cells immediately after thawing. After incubation for 30 min at 37 °C the ATP content of the cryopreserved cells recovered markedly, increasing to 76–88% of the value observed in the corresponding fresh preparation.

Discussion

A novel system developed in our laboratory enables the large scale isolation of human hepatocytes with high yield, viability and functional integrity. In the current studies we evaluated morphological and functional

characteristics of freshly isolated and cryopreserved human hepatocytes. The initial viability of freshly isolated cells was relatively high compared with other reports (Dorko et al. 1994, Hewitt et al. 1997). This is most likely due to particular modifications in the isolation method that have been applied in our system. In addition, all livers processed in this study were perfused *in situ* with cold UW solution, which preserves the organ for extended periods and enhances its viability. Thus, all livers were preserved under the best conditions.

The quantity and viability of freshly isolated and cryopreserved hepatocytes were estimated by trypan blue exclusion, according to standard procedures described for isolated hepatocytes (Berry et al. 1991). To better qualify the viability of prepared cells we used another vital stain for hepatocytes, which is labeling with a fluorescein acetate (CFSE). This dye freely passes through cell membranes and the acetyl groups are cleaved by

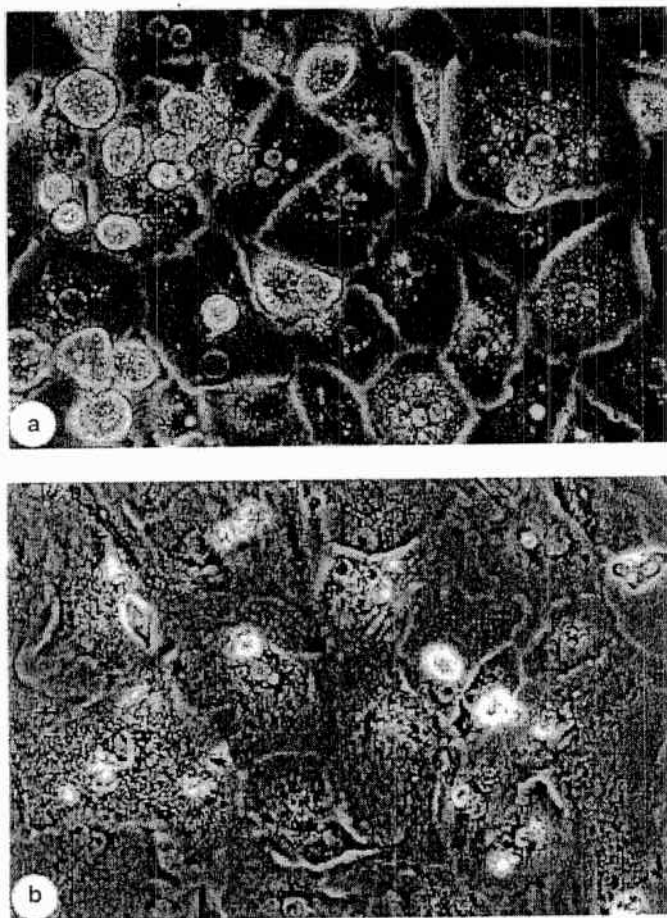


Figure 4. Morphology of freshly isolated and cryopreserved/thawed human hepatocytes cultured on collagen-coated dishes. (a) confluent culture of fresh hepatocytes approximately 12 h after seeding. (b) cryopreserved/thawed hepatocytes one week after plating. (a, b) $\times 200$.

esterase (only in viable cells). The product is fluorescent and is retained within the cell. Staining with CFSE is a relatively new method that correlates with hepatocyte function and permits the visualization of these cells by fluorescent microscopy (Soriano et al. 1992, Ostrowska et al. 1999). Although the initial viability in our study was always high, in six out of eight cases it decreased significantly following cryopreservation. Similar results were obtained with both dyes. To verify the fluorescence of CFSE-labeled hepatocytes we used a flow cytometry analysis which showed that there was no significant difference in viability of CFSE-marked and trypan blue-stained cells. Similar observations on the fluorescent staining of cells have been reported in

isolated rat hepatocytes (Nyberg et al. 1993). In our studies, we observed that trypan blue exclusion tests and CFSE labeling were also inversely correlated with LDH release measurements. LDH is a large enzyme, located almost entirely in the cytoplasm, and its leakage is a good marker of cell damage in the suspension (Berry et al. 1991). Recent studies with rat hepatocytes have shown that cold storage of cells for 48 h resulted in a greater release of this enzyme into the culture medium (Vreugdenhil et al. 1996).

Initial, crude preparations of freshly isolated hepatocytes are contaminated by dead and damaged cells as well as by nonparenchymal cells, including Kupffer cells, endothelial cells, and lymphocytes. The focus of

standard hepatocyte isolation methods is the elimination of all of these potential contaminants. The rationale for eliminating dead or damaged hepatocytes is self-evident, whereas the desirability of nonparenchymal cells is determined by the intended use of the cells. We have reported previously (Karrer et al. 1997) that modification of hepatocyte preparations, such as depletion of antigen presenting nonparenchymal cells (including passenger leukocytes) may result in ablation of the immune response, which is important for the *in vivo* alloreactivity. For this reason, we further purify all cell preparations when the initial viability is lower than 80%. In our current experiments, we purified crude hepatocytes (livers # 4, 5, 6) by a standard Percoll treatment. One good technique to check the purity of hepatocyte preparation is staining with an antibody to CD45 (leukocyte common antigen), which is expressed on the

surface of T and B lymphocytes, mononuclear phagocytes and polymorphonuclear leukocytes, but not on hepatocytes. By using this simple cell purity validation method, we have demonstrated that Percoll purification significantly reduces the contamination of hepatocyte preparations by CD45+ nonparenchymal cells.

In primary cultures, the plating efficiency and survival of freshly isolated hepatocytes depends on the experimental conditions such as soluble factors, extracellular matrix and cell-cell interactions (Guillouzo et al. 1997). Freshly isolated human hepatocytes in culture have been reported to attach well to the plates coated with collagen or Matrigel. Under appropriate culture conditions, attached hepatocytes remain viable for extended periods of time and retain a number of differentiated liver functions. However, several studies have shown that the freezing/thawing process may damage the plasma membrane integrity, transport processes, and protein synthesis in isolated hepatocytes; these effects could be critical for the ability of the cells to attach and form a monolayer (DeLoecker et al. 1990, 1993). Only a few studies have dealt with human hepatocytes after cryopreservation and variable results were obtained. The time course and plating efficiency of human hepatocytes recovered from cryopreservation have been reported to be similar to that observed using fresh hepatocytes (Chesne et al. 1993, Adams et al. 1995). Another study reported that only one out of nine human hepatocyte preparations attached on collagen-coated plastic within 2 h (Ruegg et al. 1997). Our experiments demonstrated a dramatic inhibition of hepatocyte attachment in cryopreserved/thawed preparations, even in those cases

Table 3. Plating efficiency of fresh and cryopreserved/thawed hepatocytes as determined by calculating the percentage of attached cells after 12 h incubation.

Liver #	Hepatocyte preparations	
	Plating (Fresh)	Plating (Cryopreserved)
1	92% \pm 1.7	43% \pm 1.7*
2	87% \pm 1.7	63% \pm 1.2*
3	98% \pm 0.6	72% \pm 1.2*
4	87% \pm 2.3	48% \pm 3.5*
5	90% \pm 2.9	32% \pm 2.3*
6	97% \pm 0.6	22% \pm 1.7*
7	98% \pm 0.6	22% \pm 1.7*
8	96% \pm 0.6	70% \pm 2.9*

(*) $p < 0.05$ vs. fresh hepatocytes.

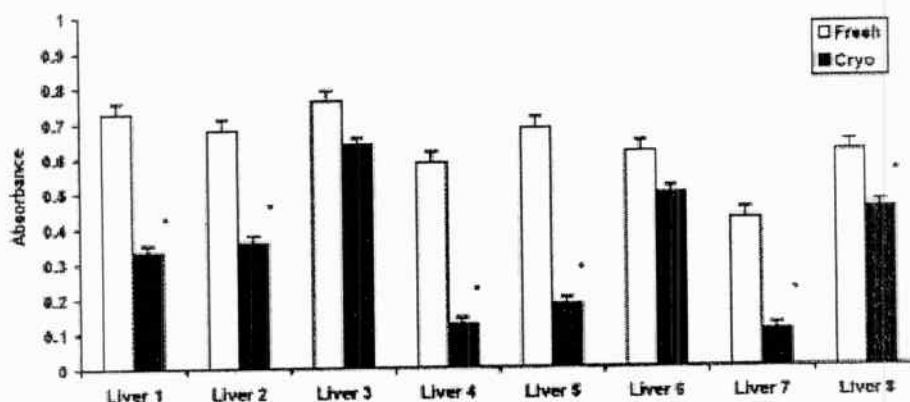


Figure 5. Viability of fresh and cryopreserved/thawed hepatocytes cultured for 12 h as evaluated by MTT colorimetric assay; (*) $p < 0.05$ vs. fresh hepatocytes.

Table 4. Metabolism of 7-ethoxycoumarin by fresh and cryopreserved/thawed hepatocytes (nmol/10⁶ cells/2 h.)

Liver #	Hepatocyte preparation							
	Fresh				Cryopreserved			
	7-HC	7-HC Gluc.	7-HC Sulf.	Total ^a	7-HC	7-HC Gluc.	7-HC Sulf.	Total
1	6.29 ± 0.42	2.58 ± 0.21	0	8.87 ± 0.19	5.84 ± 0.59	6.64 ± 0.59	0	7.48 ± 0.53
2	3.32 ± 0.27	1.16 ± 0.16	0.37 ± 0.02	4.85 ± 0.19	3.66 ± 0.20	1.68 ± 0.13	0.41 ± 0.02	5.75 ± 0.54
3	1.81 ± 0.14	0.54 ± 0.40	0	2.35 ± 0.08	1.45 ± 0.19	0.91 ± 0.17	0	2.36 ± 0.11
4	4.28 ± 0.51	5.97 ± 0.58	0	10.25 ± 0.37	3.76 ± 0.22	5.18 ± 0.35	0	8.94 ± 0.74
5	5.11 ± 0.26	3.96 ± 0.09	0.26 ± 0.01	9.33 ± 0.36	3.96 ± 0.15	3.61 ± 0.28	0.19 ± 0.01	7.76 ± 0.25*
6	2.12 ± 0.17	4.84 ± 0.16	0	6.96 ± 0.13	1.62 ± 0.09	4.29 ± 0.09	0	5.91 ± 0.37
7	4.09 ± 0.27	3.28 ± 0.18	0.18 ± 0.04	7.55 ± 0.31	3.36 ± 0.48	2.92 ± 0.11	0.25 ± 0.03	6.53 ± 0.34
8	0.86 ± 0.14	0.62 ± 0.03	0	1.48 ± 0.15	0.47 ± 0.08	0.32 ± 0.02	0	0.79 ± 0.07*

(*) $p < 0.05$ vs. fresh hepatocytes. ^asum of three metabolites. 7-HC, 7-hydroxycoumarin. 7-HC, Gluc., 7-hydroxycoumarin glucuronide. 7-HC Sulf., 7-hydroxycoumarin sulfate.

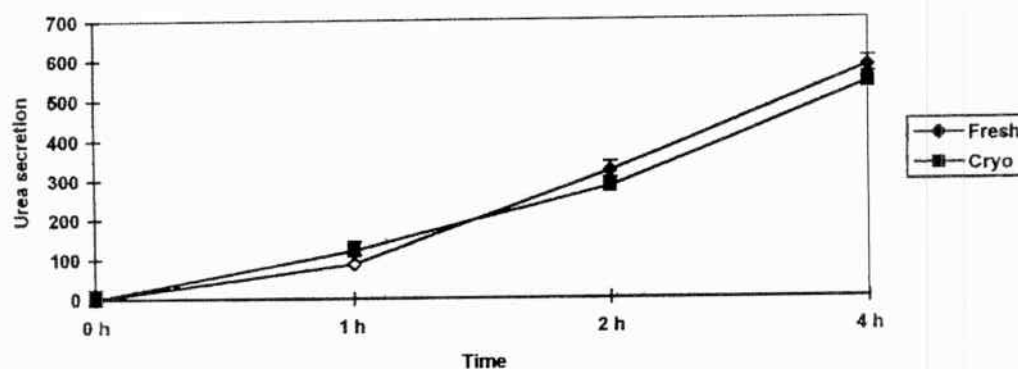


Figure 6. Average basal rates of urea synthesis for fresh and cryopreserved/thawed hepatocytes from all processed livers (nmol/10⁶ cells/4 h).

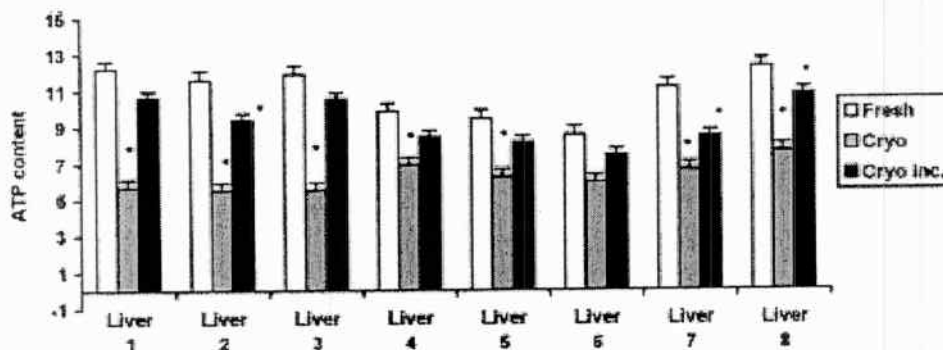


Figure 7. Energy status of freshly isolated and cryopreserved (immediately after thawing and after incubation for 30 min at 37°C) hepatocytes as evaluated by measurement of intracellular ATP content (nmol/10⁶ cells); (*) $p < 0.05$ vs. fresh hepatocytes.

where the cells showed high viability in suspension and acceptable levels of integrity. In the context of developing an optimal culture system, it is extremely difficult to obtain a confluent culture of hepatocytes recovered from cryopreservation. For studies in which maintenance of viable hepatocytes is required for an extended time (more than 5–6 h), confluent, monolayer cultures are recommended. For other applications, including investigations of drug metabolism, the function of hepatocytes recovered from cryopreservation can be successfully evaluated in the suspension cultures, where plating efficiency is not an issue.

A variety of tests are available for the detection of hepatocellular damage in culture. In this study, the functional integrity of fresh and cryopreserved/thawed hepatocytes was evaluated by the MTT assay after being cultured for 12 h in 96-well plates. MTT tetrazolium salts undergo a color change caused by the activity of mitochondrial dehydrogenases in living cells only. The number of viable hepatocytes is proportional to the concentration of the MTT reaction product, as determined by the optical density (Denizot & Lang 1986). It has been demonstrated by other authors that the MTT assay is a simple and reliable method to assess hepatocyte viability (Fuji et al. 1995, Sun et al. 1997). Although this assay is very sensitive, it depends not only on the percentage of viable cells but also on the plating efficiency of the culture. In the current studies, the reaction product was expressed more intensely in fresh preparations than in cryopreserved cells, but was generally correlated with the plating efficiency.

To estimate the functional characteristics of the hepatocytes, we assessed the rates of 7-ethoxycoumarin metabolism, ureagenesis, and intracellular ATP content in a serum-free incubation system. Deethylation of 7-EC is now widely used as a probe substrate to measure the cytochrome P450, UDP-glucuronosyltransferase and sulfotransferase activities in *in vitro* liver models. Thus, the 7-EC assay can be used to assess coupled phase I and phase II biotransformation activity (Barr et al. 1991, Walsh et al. 1995, de Kanter et al. 1998, Olinga et al. 1998). The results of the current study indicated that metabolic activity was maintained in both fresh and cryopreserved hepatocytes. However, the activity of the thawed cells was expressed at different levels. The low sulphation of 7-HC in human hepatocytes has been reported by some other authors (Ruegg et al. 1995). The almost complete absence of 7-HC sulfate formation in the *in vitro* preparations of human hepatocytes can be related with cold or warm ischemia damage, possibly because of the loss of the necessary cosubstrates (Olinga et al. 1998).

The response of isolated hepatocytes to ammonium chloride is a useful indicator of ATP synthesis. In this experiment, the process of ureagenesis was found to be well maintained in cryopreserved hepatocytes which is consistent with several other reports (Pang et al. 1997, Matsuda et al. 1999).

Intracellular ATP is used as a basic energy in all cells, including hepatocytes, and its concentration can be reduced by several factors such as donor nutritional status, cell storage conditions, and warm and cold ischemia time (Brandhorst et al. 1999). The measurement of ATP content in a cell preparation is essential to ensure that the cells are metabolically viable. A drop in the ATP content triggers a series of intracellular changes that eventually lead to irreversible cell damage. Based on our results, we conclude that cryopreservation does not cause a significant depletion of intracellular ATP. However, the recommended purification method and optimized culture conditions should be applied immediately after hepatocyte thawing in order to achieve the desired results.

To date, hepatocytes have been isolated from a variety of liver samples, including whole livers, wedge biopsy samples, and small biopsy fragments (Mito & Kusano 1993, Strom et al. 1997). This has resulted in successful therapeutic hepatocyte transplants and significant progress in bioartificial liver studies. One of the major problems limiting the application of human hepatocytes is the chronic shortage of liver donors. Thus, the ability to preserve and store these valuable cells would greatly facilitate biomedical research. Our previous experiments with rat hepatocytes demonstrated that the morphological integrity of the cryopreserved cells was strictly related to the method of preservation (Bilir et al. 1995). Although an efficient and reproducible hepatocyte cryopreservation method is enormously difficult to establish, the controlled-rate freezing technique performed in the present work is a promising method to be used in the development of large-scale human hepatocyte banking. The results of this work show that, in general, cryopreservation maintains the functional and morphological integrity of isolated hepatocytes *in vitro*. This study confirms the importance and feasibility of human hepatocyte banking.

References cited

- Adams RM, Wang M, Crane AM, Brown B, Darlington GJ and Ledley FD (1995) Effective cryopreservation and long-term storage of primary human hepatocytes with recovery of viability, differentiation, and replicative potential. *Cell Transplant* 4: 579–586

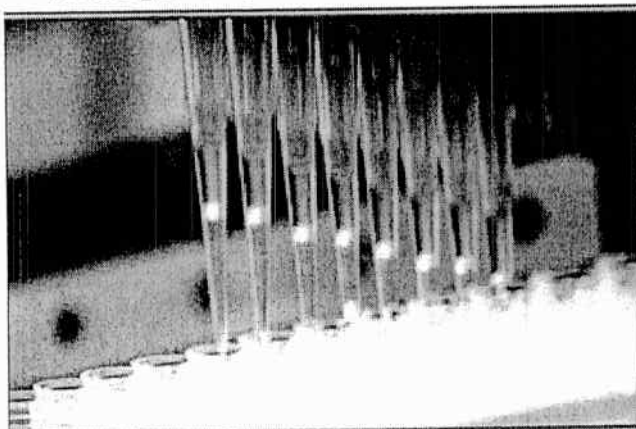
- Barr J, Weir AJ, Brendel K and Sipes IG (1991) Liver slices in dynamic organ culture. II. An *in vitro* cellular technique for the study of integrated drug metabolism using human tissue. *Xenobiotica* 21: 341-350
- Berry MN, Edwards AM and Barritt GJ (1991) Isolated hepatocytes: preparation, properties and applications. In: Burdon RH and Knippenberg PH (eds) *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol 21, pp 1-460. Elsevier, Amsterdam
- Bilir B, Ostrowska A, Hanks T and Karrer FM (1995) Controlled rate freezing of hepatocytes: a step closer to human hepatocyte banking. *Hepatology* 22(pt 2): 211A
- Bode DC (1997) Organization and activities of human tissue banks in the U.S. with special reference to the International Institute for the Advancement of Medicine. *Tiss Cult Res Commun* 16: 157-163
- Brandhorst D, Brandhorst H, Hering BJ, Federlin K and Bretzel RG (1999) Large variability of the intracellular ATP content of human islets isolated from different donors. *J Mol Med* 77: 93-95
- Chesne C, Guyomard C, Fautrel A, Poullain MG, Fremont B, De Jong H and Guillouzo A (1993) Viability and function in primary culture of adult hepatocytes from various animal species and human beings after cryopreservation. *Hepatology* 18: 406-414
- Coundouris JA, Grant MH, Engeset J, Petrie JC and Hawksworth GM (1993) Cryopreservation of human adult hepatocytes for use in drug metabolism and toxicity studies. *Xenobiotica* 23: 1399-1409
- De Loecker R, Fuller BJ, Gruwez J and De Loecker W (1990) The effects of cryopreservation on membrane integrity, membrane transport, and protein synthesis in rat hepatocytes. *Cryobiology* 27: 143-152
- De Loecker P, Fuller BJ, Koptelov VA and De Loecker W (1993) Metabolic activity of freshly prepared and cryopreserved hepatocytes in monolayer culture. *Cryobiology* 30: 12-18
- Denizot F and Lang R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods* 89: 271-277
- Dorko K, Freeswick PD, Bartoli F, Cicalese L, Bardsley BA, Tzakis A and Nussler AK (1994) A new technique for isolating and culturing human hepatocytes from whole or split livers not used for transplantation. *Cell Transplant* 3: 387-395
- Fox JJ, Chowdhury JR, Kaufmann SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV and Strom SC (1998) Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 14: 1422-1426
- Fujii T, Ha H, Yokoyama H, Hamamoto H, Yoon SH and Hori H (1995) Applications of MTT assay to primary cultured hepatocytes. *Biol Pharm Bull* 18: 1446-1449
- Guillouzo A, Morel F, Langouët, Maheo K and Rissel M (1997) Use of hepatocyte cultures for the study of hepatotoxic compounds. *J Hepatol* 26 (Suppl 2): 73-80
- Hewitt WR, Corno V, Eguchi S, Kamlot A, Middleton Y, Beeker T, Demetriou AA and Rozga J (1997) Isolation of human hepatocytes from livers rejected for whole organ transplantation. *Transplant Proc* 29: 1945-1947
- Hasset C, Laurenzana EM, Sidhu JS and Omiecinski C (1998) Effects of chemical inducers on human microsomal epoxide hydrolase in primary hepatocyte cultures. *Biochem Pharmacol* 55: 1059-1069
- Howard RB, Christensen AK, Gibbs FA and Pesch LA (1967) The enzymatic preparation of isolated intact parenchymal cells from rat liver. *J Cell Biol* 35: 675-684
- de Kanter R, Olinga P, Hof I, de Jager M, Verwillegen WA, Slooff MJ, Koster HJ, Meijer DKF and Groothuis GMM (1998) A rapid and simple method for cryopreservation of human liver slices. *Xenobiotica* 28: 225-234
- Karrer FM, Bilir B, Gill A, Ostrowska A, Stegall M, Wachs M and Kam I (1997) Cryopreserved hepatocytes are poor stimulators of *in vitro* cytotoxicity. *Transplant Proc* 29: 1111-1112
- Li AP, Reith MK, Rasmussen A, Gorski JC, Hall SD, Xu L, Kaminski D and Cheng LK (1997) Primary human hepatocytes as a tool for the evaluation of structure-activity relationship in cytochrome P450 induction potential of xenobiotics: evaluation of rifampin, rifapentine and rifabutin. *Chem Biol Interact* 107: 17-30
- Maganto P, Cienfuegos JA, Santamaria L, Rodriguez V, Eroles G, Andres S, Castillo-Olivares JL and Municio AM (1990) Auxiliary liver by transplanted frozen-thawed hepatocyte. *J Surg Res* 48: 24-32
- Matsuda H, Yagi T, Matsuoka J, Yamamura H and Tanaka N (1999) Subzero nonfreezing storage of isolated rat hepatocytes in University of Wisconsin solution. *Transplantation* 67: 186-191
- Mito M and Kusano M (1993) Hepatocyte transplantation in man. *Cell Transplant* 2: 65-74
- Nyberg SL, Shatford RA, Payne WD, Hu WS and Cerra FB (1993) Staining with fluorescein diacetate correlates with hepatocyte function. *Biotech Histochem* 68: 56-63
- Olinga P, Merema M, Hof IH, de Jong KP, Slooff MJH, Meijer DKF and Groothuis GMM (1998) Effect of human liver source on the functionality of isolated hepatocytes and liver slices. *Drug Metab Dispos* 26: 1-5
- Ostrowska A, Karrer FM and Bilir B (1999) Histological identification of purified and cryopreserved allogeneic hepatocytes following transplantation in a murine model without host immunosuppression. *Transplant Int* 12: 188-194
- Pang JM, Zaleski J and Kauffman FC (1997) Toxicity of allyl alcohol in primary cultures of freshly isolated and cryopreserved hepatocytes maintained on hydrated collagen gels. *Toxicol Appl Pharmacol* 142: 87-94
- Powis G, Melder DC and Wilke TJ (1989) Human and dog, but not rat, isolated hepatocytes have decreased foreign compound-metabolizing activity compared to liver slices. *Drug Metab Dispos* 17: 526-531
- Ruegg CE, Silber PM, Mughal RA, Ismail J, Lu C, Bode DC and Li AP (1997) Cytochrome-P450 induction and conjugated metabolism in primary human hepatocytes after cryopreservation. *In Vitro Toxicol* 10: 217-222
- Seglen PO (1976) Preparation of isolated rat liver cells. *Methods Cell Biol* 13: 29-83
- Soriano HE, Lewis D, Legner M, Brandt M, Baley P, Darlington G, Finegold M and Ledley FD (1992) The use

- of Dil-marked hepatocytes to demonstrate orthotopic, intra-hepatic engraftment following hepatocellular transplantation. *Transplantation* 54: 717-723
- Strom SC, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM and Posner MP (1997) Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. *Transplantation* 63: 559-569
- Strom SC, Chowdhury JR and Fox IJ (1999) Hepatocyte transplantation for the treatment of human disease. *Semin Liv Dis* 19: 39-48
- Sun J, Wang L, Waring MA, Wang C, Woodman KK and Sheil AG (1997) Simple and reliable methods to assess hepatocyte viability in bioartificial liver support system matrices. *Artif Organs* 21: 408-413
- Vreugdenhil PK, Marsh DC and Southard JH (1996) Comparison of isolated hepatocytes and tissue slices for study of liver hypothermic preservation/reperfusion injury. *Cryobiology* 33: 430-435
- Walsh JS, Patanella JE, Halm KA and Facchine KL (1995) An improved assay for the assessment of liver slice metabolic viability using 7-Ethoxycoumarin. *Drug Metab Dispos* 23: 869-874
- Wroblewski F and LaDue JS (1955) Lactic dehydrogenase activity in blood. *Proc Soc Exp Biol Med* 90: 210

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Metabolism-Based Drug-Drug Interaction Studies

Due to the possibility of associated drug interactions and hepatotoxicity, the USFDA has placed new emphasis on metabolic drug-drug interaction studies for determining whether a new chemical entity (NCE) is likely to affect the metabolic elimination of drugs already on the market or vice versa. To address this requirement, CellzDirect offers CYP450 induction, CYP450 inhibition, and *in vitro* phase IV drug interaction studies.

Cytochrome P450 (CYP450) Induction

CYP450 enzymes can be induced as a result of drug exposure, which may cause increased formation of toxic metabolites and/or decreased systemic levels of a co-administered drug, and accordingly make that drug less effective or more toxic. Primary cultures of human hepatocytes are widely accepted as the gold standard for determining enzyme induction potential of drugs *in vitro*. CellzDirect's scientific staff have been instrumental in advancing the science and technologies relating to the use of human hepatocytes for assessing enzyme induction.

In a typical study, fresh human hepatocytes from three separate donors are exposed daily for 2-3 days with test article at clinically-relevant concentrations. Drug-induced changes in CYP 450 immunoreactive protein or mRNA are measured by western immunoblot or quantitative PCR (Taqman®), respectively. Changes in CYP450 enzyme activities are determined either by placing probe substrates directly onto intact monolayers or using isolated microsomes. The relative potency of a NCE is compared to that of known positive controls in accordance with the USFDA guidance on drug-drug interactions.

Ex vivo analysis of enzyme induction can also be performed by measuring cytochrome P450 activities in microsomes prepared from liver tissue harvested from animals treated with an NCE and appropriate controls.

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CYP450 Inhibition (Direct and Time-Dependent)

Inhibition of a specific drug-metabolizing enzyme can decrease the metabolic clearance of a co-administered drug leading to elevated blood concentrations, which may cause toxicity or enhanced effects. In an IND submission, the USFDA requires the determination of the potential for a drug candidate to inhibit CYP450 enzymes.

Inhibition of CYP450 activity can be evaluated by incubating a NCE at multiple concentrations with pooled human microsomes or hepatocytes and known CYP450-specific substrates. The effect of the NCE on turnover of CYP450-specific substrates is determined by LC-MS/MS analysis of metabolite formation, and an IC_{50} value is determined. For assessment of time-dependent inhibition a pre-incubation of the test compound with pooled microsomes in the presence and absence of NADPH is performed prior to determining the effect on turnover of probe substrates. If significant inhibition (i.e. $IC_{50} \leq 50 \mu M$) is observed after initial evaluation, then the K_i (or k_{inact} and K_i for time-dependent inhibition) for the relevant CYP450 isoforms is typically determined.

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***In Vitro* Phase IV Drug Interaction Studies**


Drug interactions involving a NCE may lead to changes in the metabolism of co-administered compounds. Likewise, inhibition by a co-administered compound may lead to changes in the metabolism of the NCE. If these interactions are not identified early in drug development, the result can be exposure to toxic drug levels in some patients, resulting in a "black box" warning on the product label or withdrawal from the market.

In a typical *in vitro* Phase IV study, the rate of disappearance of certain marketed compounds is measured in the presence and absence of a NCE in human liver microsomes or hepatocytes. The effect of administering the marketed compounds with the test compound also is examined by monitoring the disappearance of the test compound in the same incubates. The goal is to predict whether patients taking the marketed compounds might be susceptible to a metabolic interaction with the test compound. To this end, methods can be developed and validated for each marketed compound in human hepatocytes. Concentrations of the marketed compounds and test compound are chosen based on relevant clinical or PK data.

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Metabolism-Based Drug-Drug Interaction Studies As Described by the Draft FDA Guidance (2006)

"Many metabolic routes of elimination, including most of those occurring through the P450 family of enzymes, can be inhibited or induced by concomitant drug treatment. Observed changes arising from metabolic drug-drug interactions can be substantial — an order of magnitude or more decrease or increase in the blood and tissue concentrations of a drug or metabolite — and can include formation of toxic and/or active metabolites or increased exposure to a toxic parent compound. These large changes in exposure can alter the safety and efficacy profile of a drug and/or its active metabolites in important ways. This is most obvious and expected for a drug with a narrow therapeutic range (NTR), but is also possible for non-NTR drugs as well (e.g., HMG CoA reductase inhibitors). It is important that metabolic drug-drug interaction studies explore whether an investigational agent is likely to significantly affect the metabolic elimination of drugs already in the marketplace and likely in medical practice to be taken concomitantly and, conversely, whether drugs in the marketplace are likely to affect the metabolic elimination of the investigational drug. Even drugs that are not substantially metabolized can have important effects on the metabolism of concomitant drugs. For this reason, metabolic drug-drug interactions should be explored, even for an investigational compound that is not eliminated significantly by metabolism."

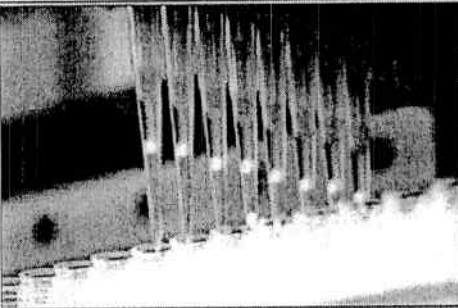


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Drug Metabolism Studies

Because metabolites can have a major affect on how a drug or combination of drugs are metabolized and eliminated, the FDA has determined a drug assessment should include a description of its metabolism and how it may affect overall elimination. CellzDirect offers studies designed to determine metabolic stability and metabolite profiling for species comparison.

Metabolic Stability

Estimates of *in vivo* metabolic drug clearance can be determined from *in vitro* metabolism kinetic data. Metabolic stability studies are typically performed to estimate a drug candidate's metabolic half-life and intrinsic clearance rates which can be major determinants for *in vivo* drug efficacy. Compounds with short half-lives may require multiple doses to maintain effective plasma levels, whereas compounds with longer half-lives and slower elimination kinetics may require complicated dosing regimens.

For typical studies, two concentrations of the test article are incubated with pooled microsomes and/or hepatocytes for 0, 10, 20, 30 and 60 minutes or 0, 30, 60, 120 and 240 minutes, respectively. Metabolic turnover is determined by loss of parent compound as measured by LC-MS/MS or HPLC and the half-life ($t_{1/2}$) of the test article is estimated.

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Metabolic Profiling/Species Comparison

A description of all major circulating metabolites is necessary to assess the safety and effectiveness of a drug. Metabolic profiling aims to profile major metabolites of drug candidates that are formed in humans and compares the profiles for human and animal *in vitro* model systems. This information is then used to choose a rodent or non-rodent species for *in vivo* toxicity and pharmacokinetic studies.

The most complete picture of hepatic metabolism can be obtained with intact hepatocytes which provide cellular integrity with respect to enzyme architecture and phase II metabolism and potentially allow for any concentration gradients mediated by transporters that may affect exposure of drugs to enzymes. Other *in vitro* models typically utilized for metabolite profiling include subcellular fractions such as microsomes, S9, and cytosol.

Human and animal hepatocytes and/or microsomes are incubated with test article for multiple time points. The metabolism of the parent compound and appearance and characterization of metabolites are determined by HPLC and LC-MS/MS methods. Then, the metabolite profile for human is compared to animal species such as mouse, rat, dog and non-human primate.

RapidAlert



Drug Metabolism Studies Based on the FDA Draft Guidance (2006)

"The desirable and undesirable effects of a drug arising from its concentrations at the sites of action are usually related either to the amount administered (dose) or to the resulting blood concentrations, which are affected by its absorption, distribution, metabolism, and/or excretion. Elimination of a drug or its metabolites occurs either by metabolism, usually by the liver or gut mucosa, or by excretion, usually by the kidneys and liver. In addition, protein therapeutics may be eliminated through a specific interaction with cell surface receptors, followed by internalization and lysosomal degradation within the target cell. Hepatic elimination occurs primarily by the cytochrome P450 family (CYP) of enzymes located in the hepatic endoplasmic reticulum, but may also occur by non-P450 enzyme systems, such as N-acetyl and glucuronosyl transferases. Many factors can alter hepatic and intestinal drug metabolism, including the presence or absence of disease and/or concomitant medications, or even some foods, such as grapefruit juice. While most of these factors are usually relatively stable over time, concomitant medications can alter metabolism abruptly and are of particular concern. The influence of concomitant medications on hepatic and intestinal metabolism becomes more complicated when a drug, including a prodrug, is metabolized to one or more active metabolites. In this case, the safety and efficacy of the drug/prodrug are determined not only by exposure to the parent drug but by exposure to the active metabolites, which in turn is related to their formation, distribution, and elimination. Therefore, adequate assessment of the safety and effectiveness of a drug includes a description of its metabolism and the contribution of metabolism to overall elimination. For this reason, the development of sensitive and specific assays for a drug and its important metabolites is critical to the study of metabolism and drug-drug interactions."

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PREDICTION OF HEPATIC CLEARANCE AND AVAILABILITY BY CRYOPRESERVED HUMAN HEPATOCYTES: AN APPLICATION OF SERUM INCUBATION METHOD

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ABSTRACT:

A novel and convenient method was established for the prediction of *in vivo* metabolic clearance in human liver. The present method applied the *in vitro-in vivo* extrapolation paradigm previously established in rats to the *in vitro* data obtained from cryopreserved human hepatocytes. Predicted hepatic availability and clearance were compared with the reported oral bioavailability and plasma clearance in humans for 14 clinically used drugs (naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, timolol, antipyrine, diazepam, quinidine, caffeine, propranolol, diclofenac, and phenacetin). A large interindividual variation was observed in the intrinsic metabolic clearance among separate cryopreserved preparations from different subjects. The prediction generally resulted in a marked underestimation when the biologically based scaling

factor (3.1×10^9 cells/kg) was used for the extrapolation of *in vitro* data (milliliters per minutes per cells) to *in vivo* value (milliliters per minutes per kilograms). Reasonably good *in vitro-in vivo* correlations were obtained with empirically calculated scaling factors, 8.5×10^9 (cells/kg) from 10 individual preparations and 10.8×10^9 (cells/kg) from pooled preparation of two selected lots, which were 3- to 4-fold larger than the biologically based scaling factor. These data suggested that the calibration of inherent interindividual variation of metabolic activities among different cryopreserved preparations of human hepatocytes to obtain the empirical scaling factor, which is applicable only to the preparation used, was an essential step for more reliable and quantitative prediction of *in vivo* metabolic activity in humans.

Hepatic clearance for the metabolism of compounds kinetically consists of two major determinants: intrinsic (metabolic) clearance of the unbound compound and unbound fraction of compound in the blood (or plasma when corrected by the blood-to-plasma partition). Generally, the intrinsic clearance for the unbound compound is measured *in vitro* by the incubation of isolated hepatocytes or subcellular fractions such as S-9 and microsomes in the protein-free medium. The *in vitro* metabolic parameters thus obtained are extrapolated by using anatomical parameters such as cell numbers and protein content in the intact liver for the prediction of *in vivo* metabolic activity (Houston and Carlile, 1997; Iwatsubo et al., 1997; Obach, 1999). Separate experiments necessarily are further carried out to measure the unbound fraction in the plasma. Many technical problems including adsorption of compounds to the apparatus during the equilibrium dialysis and ultra-filtration often hamper the accuracy of the evaluated values (Bertilsson et al., 1979; Desoye, 1988). To improve the accuracy and avoid complexity for predicting *in vivo* metabolic clearance from *in vitro* experiments, we have recently developed a novel and convenient *in vitro* method for predicting *in vivo* metabolic clearance by using freshly isolated rat hepatocytes suspended in rat serum (Shibata et al., 2000). Oral bioavailability and hepatic clearance for 16 widely used compounds were well predicted directly from the *in vitro* metabolic clearance values obtained from a single incubation without separate evaluation of unbound fraction in the plasma. The purposes

of the present study were to 1) determine whether the same methodology was applicable to the prediction of *in vivo* metabolic activity in humans by using cryopreserved human hepatocytes and 2) establish the *in vitro-in vivo* scaling-up paradigm to calibrate the interindividual variation of the metabolic activities among cryopreserved preparations from different subjects for more reliable and quantitative prediction in humans.

Materials and Methods

Naloxone and lidocaine were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. Buspirone, metoprolol, phenacetin, propranolol, quinidine, timolol, and verapamil were obtained from Sigma-Aldrich (St. Louis, MO). Antipyrine, caffeine, diazepam, diclofenac, and imipramine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Blood was collected from three healthy male volunteers aged 25 to 40 years old and allowed to coagulate for 3 h at room temperature. The blood was later centrifuged (15 min, 1800g) to obtain serum. The serum was stored at -80°C until use. The pH of human serum was adjusted to 7.4 at 37°C by adding 1N-HCl solution before use. Cryopreserved human hepatocytes (lot numbers 56, 57, 64, 70, 73, 83, 97, 100, 106, and 120) were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Cell viability was assessed using the 0.4% trypan blue exclusion test, and the count of living cells was started 5 min after mixing the pigment. Cell viabilities were between 45 and 60%. Hepatocytes were resuspended in 100% human serum at an ice-cold temperature at the following densities: 1×10^6 cells/ml for naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, and timolol; 2×10^6 cells/ml for quinidine, caffeine, propranolol, diclofenac, and phenacetin; and 5×10^6 cells/ml for antipyrine and diazepam. Suspensions of hepatocytes (370 μl) were pipetted into 1.5-ml tubes, and an aliquot (3.7 μl) of 100 μM compound in a water (or 50% CH_3CN for quinidine and phenacetin) was added to obtain the final concentration of 1 μM (or 50 μM for

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antipyrine and 10 μ M for caffeine). Each sample (50 μ l) was transferred to two 96-well plates with flat bottoms ($n = 3$), each of which was used for the incubation or for the control. Ninety-six well plates were incubated at 37°C with shaking at 150 rpm under 95% O₂/5% CO₂ in the water bath incubator. After the onset of incubation, the plates were placed on ice at the designated time point, and the reaction was terminated by the addition of a 150- μ l ice-cold ethanol solution containing the internal standard (no internal standard for antipyrine and caffeine). The sample was centrifuged (10,000g \times 10 min), and the amount of compound remaining in the supernatant was measured by HPLC¹-UV (antipyrine and caffeine) or LC-MS/MS (others) as described below.

Antipyrine was measured at 254 nm by the Alliance 2690–2487 HPLC-UV system (Waters Corp., Milford, MA) that was connected to Inertsil ODS-3 4.6 \times 250 mm (GL-Sciences, Tokyo, Japan), which acted as an analytical column. The HPLC method involved the isocratic elution with acetonitrile/water (25:75) containing 10 mM ammonium acetate at the flow rate of 1 ml/min. The retention time of antipyrine was 7 min. Caffeine was measured by the UV wavelength of 280 nm at a flow rate of 1 ml/min of acetonitrile/water (10:90) containing 0.1% trifluoroacetic acid. The retention time of caffeine was 16 min. Other compounds were measured by the Alliance HT 2790 HPLC (Waters Corp.), PU-1580 HPLC pump (Jasco, Tokyo, Japan), NANOSPACE SI-2 switching valves (Shiseido, Osaka, Japan), and API-3000 LC-MS/MS detector (PerkinElmerSciex Instruments, Boston, MA) with a turbo ionspray interface. Multiple reaction monitoring of positive-ion mode was used for all analyses. Analytical methods including the selection of ions and parameters for multiple reaction monitoring were automatically obtained for each compound by the application software, Analyst (PerkinElmerSciex Instruments). Mass number of molecular ions and product ions for each compound was identified as follows (molecular > product): naloxone 328.4 > 310.4, buspirone 386.2 > 122.2, verapamil 455.3 > 165.2, lidocaine 235.2 > 86.2, imipramine 281.1 > 86.0, metoprolol 268.3 > 116.0, timolol 317.1 > 261.1, diazepam 285.1 > 193.3, quinidine 325.1 > 307.3, propranolol 260.2 > 116.3, diclofenac 295.9 > 215.2, and phenacetin 180.1 > 138.0. A fast-gradient condition using two switching valves and pumps (3.5 min/cycle) was used for the analysis. Capcell Pak UG-120 4.0 \times 10 mm (Shiseido) was used as an analytical column, and the flow rate of 1 ml/min of acetonitrile/water (10:90) containing 10 mM ammonium acetate was the initial condition used. After the injection of a sample (5 μ l), the ratio of acetonitrile/water was changed to 90:10 linearly for 1 min and maintained for the next 0.5 min. The column was then washed with acetonitrile/water (90:10) containing 10 mM ammonium acetate at a back flow rate of 1 ml/min. The effluent was split with 0.2 ml/min, and only the effluent from 0.5 to 1.5 min after the injection was introduced into the LC-MS/MS detector. Modified conditions were used for metoprolol, timolol, and phenacetin. In the case of metoprolol and timolol, Symmetry Shield RP18/3.5 μ M 2.1 \times 10 mm (Waters Corp.) was used as the analytical column.

After the injection of a sample, the ratio of acetonitrile/water was changed linearly to 66:34 for 1.4 min, and the effluent from 0.4 to 1.5 min after the injection was introduced into the LC-MS/MS detector. In the case of phenacetin, Inertsil ODS-3 2.1 \times 150 mm (GL-Sciences) was used as the analytical column. After the injection of a sample, the ratio of acetonitrile/water was changed linearly to 34:66 for 3 min and then changed linearly to 90:10 for the next 2 min. The effluent from 0.5 to 5 min after the injection was introduced into the LC-MS/MS detector. Diazepam was commonly used as the internal standard. When diazepam was the analyte, quinidine was used as the internal standard.

For standard compounds, the following assumptions were reasonably applied to the prediction, 1) the hepatic metabolism is the major route of elimination, 2) all metabolic enzymes in the cryopreserved preparation of human hepatocytes remain active comparably to in vivo, and 3) the absorption is complete for all standard compounds. The in vitro intrinsic clearance ($CL_{int, in vitro}$) was calculated from the following equation by using cell density (D), incubation time (T , 120 min for the calculation of $CL_{int, in vitro}$), and the ratio (R) of unchanged compound concentration at time T to that at time 0 when the unbound drug concentration was much lower than its K_m value (Shibata et al., 2000); $CL_{int, in vitro} = (-\log_e R)/(D \times T)$. To extrapolate the in vitro clearance to the in vivo value, the empirical scaling factor (average SF_{70+73}) for the optimized cryopreserved preparation pooled from equal volumes of human hepatocytes (lot numbers 70 and 73) was calculated according to the method described under the Results section. The value of average SF_{70+73} was 10.8×10^9 cells/kg of body weight and used for the extrapolation as follows: $CL_{H, int, in vitro, 70+73} = CL_{int, in vitro, 70+73} \times \text{average } SF_{70+73}$ where $CL_{H, int, in vitro, 70+73}$ and $CL_{int, in vitro, 70+73}$ represent the in vitro hepatic intrinsic clearance and in vitro intrinsic clearance, respectively, measured in the pooled preparation of lot 70 and 73. We chose the dispersion model as a liver model because a good predictability of hepatic availability (F_H) for high clearance drugs was previously reported (Iwatsubo et al., 1997). The hepatic clearance ($CL_{H, predicted, 70+73}$) was predicted from the obtained in vitro hepatic intrinsic clearance ($CL_{H, int, in vitro, 70+73}$) by using the following equation (eq. 1) with the dispersion model (Iwatsubo et al., 1997);

$$CL_{H, predicted, 70+73} = Q_H \times R_B \times (1 - 4a/((1+a)^2 \times \exp[(a-1)/(2 \times D_N)] - (1-a)^2 \times \exp[-(a+1)/(2 \times D_N)]))$$

where $R_B = (CL_{H, int, in vitro, 70+73})/(Q_H \times R_B)$ and $a = (1 + 4 \times R_B \times D_N)^{0.5}$

F_H was further calculated from $F_H = 1 - \text{hepatic extraction ratio } (E_H) = 1 - CL_{H, predicted, 70+73}/(Q_H \times R_B)$. In these equations, the liver blood flow rate (Q_H) and dispersion number (D_N) for humans were assumed to be 20.7 ml/min/kg (Davies and Morris, 1993) and 0.17 (Roberts and Rowland, 1986), respectively. The blood-to-plasma concentration ratio (R_B) was used as reported or assumed to be unity if the value was not available.

Results

Tables 1 and 2 summarize the pharmacokinetic profiles in humans and the results of extrapolations from the in vitro data for the standard compounds tested in the present study. These compounds were chosen to represent a wide range of oral bioavailability (2–96%) and plasma clearance (0.3–28.3 ml/min/kg). These standard compounds are reported to have complete absorption, negligible urinary excretion (<20% of dose), and the major route of elimination by hepatic metabolism. Therefore, it was reasonably assumed that the in vivo plasma clearance ($CL_{P, in vivo}$) and oral bioavailability ($F_{PO, in vivo}$) are equal to the hepatic metabolic clearance (CL_H) and F_H , respectively. In vivo values for hepatic intrinsic clearance ($CL_{H, int, in vivo}$) of standard compounds were calculated from $F_{PO, in vivo}$ by the dispersion model using the iterative calculation method (Goal Seek method in Microsoft Excel). To calculate empirical scaling factor from the comparison of in vivo and in vitro hepatic intrinsic clearance, $CL_{int, in vitro}$ values were evaluated in the cryopreserved preparations of human hepatocytes obtained from 10 different subjects. An approximately 3- to 5-fold variation in the $CL_{int, in vitro}$ was observed

¹ Abbreviations used are: HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; $CL_{int, in vitro}$, in vitro intrinsic clearance observed when test compounds were metabolized by human hepatocytes suspended in human serum; D , cell density of hepatocytes suspended in serum; T , incubation time; R , ratio of intact drug concentration after incubation to that at time 0; SF_{70+73} , scaling factor calculated from $CL_{int, in vitro, 70+73}/CL_{H, int, in vivo}$ for the pooled hepatocyte preparation of lot 70 and 73; $CL_{H, int, in vitro, 70+73}$, hepatic intrinsic clearance calculated from in vitro data using the pooled hepatocyte preparation of lot 70 and 73; $CL_{int, in vitro, 70+73}$, in vitro intrinsic clearance observed when test compounds were metabolized in the pooled hepatocyte preparation of lot 70 and 73 suspended in human serum; F_H , hepatic availability; $CL_{H, predicted, 70+73}$, predicted hepatic clearance from $CL_{int, in vitro, 70+73}$ and average SF_{70+73} ; Q_H , hepatic blood flow rate; R_B , blood-to-plasma concentration ratio; D_N , dispersion number; $CL_{P, in vivo}$, in vivo plasma clearance; $F_{PO, in vivo}$, oral bioavailability in humans; $CL_{H, int, in vivo}$, hepatic intrinsic clearance calculated from $F_{PO, in vivo}$ by the dispersion model (using the Goal Seek method attached to Microsoft Excel); SF_{mean} , mean of scaling factor calculated from $CL_{int, in vitro, mean}/CL_{H, int, in vivo}$ for 10 individual lots; average SF_{mean} , average value of SF_{mean} for seven standard compounds; $F_H, predicted, 70+73$, predicted hepatic availability from $CL_{int, in vitro, 70+73}$ and average SF_{70+73} ; SF_{bio} , biologically based scaling factor of hepatocellularity (3.1×10^9 cells/kg); average SF_{70+73} , average value of SF_{70+73} for seven standard compounds.

TABLE 1
In vitro-in vivo correlation of seven compounds using 10 individually prepared cryopreserved human hepatocytes and key parameters for in vivo prediction

All these values were quoted from the literature as follows. Naloxone [Asali and Brown (1984); Holford (1998)]; buspirone [Gammans et al. (1986)]; verapamil [Gross et al. (1988)]; metoprolol [Johansson et al. (1974)]; lidocaine [Wing et al. (1984); Remmel et al. (1991)]; imipramine [the pharmacokinetics were mean value of the report of Nagy and Johansson (1975) and Ciraulo et al. (1988)]; metoprolol [Johansson et al. (1974)]; Regardh et al. (1981)]; timolol [Wilson et al. (1982)]; Holford (1998)].

No.	compound	f_u	R_B	$CL_{P, in vivo}$ ml/min/kg	$F_{PO, in vivo}$	$CL_{H, in vivo}$ ml/min/kg	$CL_{int, in vitro, mean}$ ml/min/10 ⁶ cells	SF 10 ⁶ cells/kg	$CL_{H, predicted, mean}$ ml/min/kg	F_H , predicted, mean	$CL_{H, predicted, bio}$ ml/min/kg	F_H , predicted, bio
1	Naloxone	0.559	1.22*	24.8	0.02	154.9	34.7 ± 8.2	4.5	25.3 ± 0.1	0.00 ± 0.00	23.8 ± 0.7	0.06 ± 0.03
2	Buspirone	0.050	0.81*	28.3 ± 10.3	0.04 ± 0.04	79.1	12.1 ± 6.9	6.5	16.2 ± 0.6	0.04 ± 0.03	13.2 ± 1.9	0.22 ± 0.11
3	Verapamil	0.100	0.77	11.8 ± 5.0	0.20 ± 0.12	31.0	7.3 ± 3.6	4.3	14.5 ± 0.9	0.09 ± 0.05	10.6 ± 1.9	0.34 ± 0.12
4	Lidocaine	0.296	0.84	12.5 ± 1.5	0.24 ± 0.05	29.8	2.3 ± 0.7	12.9	10.6 ± 1.9	0.39 ± 0.11	5.5 ± 1.4	0.68 ± 0.08
5	Imipramine	0.185	1.08	11.8 ± 8.1	0.42 ± 0.08	21.8	1.5 ± 0.6	14.5	8.6 ± 2.7	0.61 ± 0.12	4.0 ± 1.4	0.82 ± 0.06
6	Metoprolol	0.883	1.13	10.8 ± 1.5	0.50 ± 0.11	17.8	2.3 ± 0.8	7.8	12.0 ± 2.5	0.49 ± 0.10	5.8 ± 1.6	0.75 ± 0.07
7	Timolol	0.400	0.84*	7.7 ± 1.2	0.61 ± 0.06	9.1	1.0 ± 0.6	9.0	6.0 ± 2.8	0.65 ± 0.16	2.7 ± 1.4	0.84 ± 0.08
				average SF _{mean}		8.5 ± 4.0						

f_u , unbound fraction in plasma; R_B , blood-to-plasma concentration ratio (reported); $CL_{P, in vivo}$, plasma clearance in humans (reported); $F_{PO, in vivo}$, oral bioavailability in humans (reported); $CL_{H, in vivo}$, hepatic intrinsic clearance values calculated from $F_{PO, in vivo}$ by the dispersion model (using the Goal Seek method attached to Microsoft Excel); $CL_{int, in vitro, mean}$, in vitro intrinsic clearance observed when test compounds were metabolized by 10 individual lots of human hepatocytes suspended in human serum; SF_{mean}, mean of scaling factor calculated from $CL_{int, in vitro, mean} / CL_{H, in vivo}$ for 10 individual lots; $CL_{H, predicted, mean}$, predicted hepatic clearance from $CL_{int, in vitro, mean}$ and average SF_{mean} (8.5×10^6 cells/kg) as a scaling factor; F_H , predicted, mean, predicted hepatic availability from $CL_{H, predicted, mean}$ and average SF_{mean} (8.5×10^6 cells/kg) as a scaling factor; $CL_{H, predicted, bio}$, predicted hepatic clearance from $CL_{int, in vitro, mean}$ and biologically based scaling factor of hepatocellularity (SF_{bio} = 3.1×10^6 cells/kg); F_H , predicted, bio, predicted hepatic availability from $CL_{H, predicted, bio}$ and average SF_{bio} (3.1×10^6 cells/kg).

* In house data.

TABLE 2

Profiles of 14 compounds tested with the pooled preparation of human hepatocytes lot 70 and 73 and the key parameters for the in vivo prediction

All these values were quoted from the literature as follows. Naloxone [Asali and Brown (1984); Holford (1998)]; buspirone [Gammans et al. (1986)]; verapamil [Gross et al. (1988)]; metoprolol [Johansson et al. (1974)]; lidocaine [Remmel et al. (1991)]; Wing et al. (1984)]; imipramine [the pharmacokinetics were mean value of the report of Ciraulo et al. (1988) and Nagy and Johansson (1975)]; diazepam [Dvornik et al. (1983)]; quinine [Guentert et al. (1979)]; Hardy and Schentag (1988); Hughes et al. (1982)]; phenacetin [Rauha and Dobach (1975)]; Vesell et al. (1975)].

No.	compound	f_u	R_B	$CL_{P, in vivo}$ ml/min/kg	$F_{PO, in vivo}$	$CL_{H, in vivo}$ ml/min/kg	D 10 ⁶ cells/ml	R after 2-h	$CL_{int, in vitro, 70+73}$ ml/min/10 ⁶ cells	SF ₇₀₊₇₃ 10 ⁶ cells/kg	$CL_{H, predicted, 70+73}$ ml/min/kg	F_H , predicted, 70+73
1	Naloxone	0.559	1.22*	24.8	0.02	154.9	1.0	0.07 ± 0.01	18.31 ± 0.69	8.5	25.2 ± 0.0	0.01 ± 0.00
2	Buspirone	0.050	0.81*	28.3 ± 10.3	0.04 ± 0.04	79.1	1.0	0.33 ± 0.01	7.66 ± 0.26	10.3	16.5 ± 0.0	0.02 ± 0.00
3	Verapamil	0.100	0.77	11.8 ± 5.0	0.20 ± 0.12	31.0	1.0	0.51 ± 0.01	4.65 ± 0.13	6.7	14.9 ± 0.1	0.07 ± 0.00
4	Lidocaine	0.296	0.84	12.5 ± 1.5	0.24 ± 0.05	29.8	1.0	0.70 ± 0.03	2.43 ± 0.26	12.3	13.4 ± 0.6	0.23 ± 0.03
5	Imipramine	0.185	1.08	11.8 ± 8.1	0.42 ± 0.08	21.8	1.0	0.77 ± 0.01	1.78 ± 0.06	12.3	13.3 ± 0.3	0.40 ± 0.01
6	Metoprolol	0.883	1.13	10.8 ± 1.5	0.50 ± 0.11	17.8	1.0	0.81 ± 0.05	1.47 ± 0.39	12.1	12.1 ± 2.0	0.49 ± 0.09
7	Timolol	0.400	0.84*	7.7 ± 1.2	0.61 ± 0.06	9.1	1.0	0.91 ± 0.03	0.69 ± 0.23	13.2	6.5 ± 1.7	0.62 ± 0.10
8	Anipyrine	0.970	0.71	0.7 ± 0.1	0.96 ± 0.06	0.8	5.0	0.98 ± 0.01	0.03 ± 0.02		0.4 ± 0.2	0.98 ± 0.01
9	Diazepam	0.013	0.71	0.3 ± 0.1	0.94 ± 0.20	0.9	2.0	0.96 ± 0.02	0.05 ± 0.03		0.7 ± 0.4	0.95 ± 0.03
10	Quinine	0.146	0.92	4.9 ± 1.6	0.70 ± 0.17	7.1	2.0	0.84 ± 0.04	0.61 ± 0.14		6.1 ± 1.2	0.88 ± 0.06
11	Caffeine	0.650	0.89*	1.0 ± 0.4	0.92 ± 0.04	1.7	2.0	0.96 ± 0.01	0.13 ± 0.03		1.6 ± 0.4	0.92 ± 0.02
12	Propranolol	0.123	0.89*	17.3 ± 2.0	0.32 ± 0.04	24.4	2.0	0.77 ± 0.17	2.28 ± 0.07		13.6 ± 0.2	0.26 ± 0.01
13	Diclofenac	0.003	0.55	4.1 ± 0.8	0.58 ± 0.14	6.7	2.0	0.80 ± 0.04	0.52 ± 0.01		6.2 ± 0.8	0.46 ± 0.07
14	Phenacetin	0.600	1.01*	19.6 ± 4.5	0.02 ± 0.03	127.5	2.0	0.01 ± 0.00	14.72 ± 0.29		20.8 ± 0.0	0.01 ± 0.00
				average SF ₇₀₊₇₃		10.8 ± 2.4						

f_u , unbound fraction in plasma; R_B , blood-to-plasma concentration ratio (reported); $CL_{P, in vivo}$, plasma clearance in humans (reported); $F_{PO, in vivo}$, oral bioavailability in humans (reported); $CL_{H, in vivo}$, hepatic intrinsic clearance values calculated from $F_{PO, in vivo}$ by the dispersion model (using the Goal Seek method attached to Microsoft Excel); D , cell density of hepatocytes suspended in serum; R , the ratio of unchanged compound concentration at 2-hr incubation to that at time 0; $CL_{int, in vitro, 70+73}$, in vitro intrinsic clearance observed when test compounds were metabolized in the pooled preparation of Lot 70 and 73 suspended in human serum; SF₇₀₊₇₃, scaling factor calculated from $CL_{int, in vitro, 70+73} / CL_{H, in vivo}$ for pooled preparation of Lot 70 and 73; $CL_{H, predicted, 70+73}$, predicted hepatic clearance from $CL_{int, in vitro, 70+73}$ and average SF₇₀₊₇₃ (10.8×10^6 cells/kg) as a scaling factor; F_H , predicted, 70+73, predicted hepatic availability from $CL_{H, predicted, 70+73}$ and average SF₇₀₊₇₃ (10.8×10^6 cells/kg) as a scaling factor.

* In house data.

PREDICTION OF METABOLIC CLEARANCE IN HUMANS

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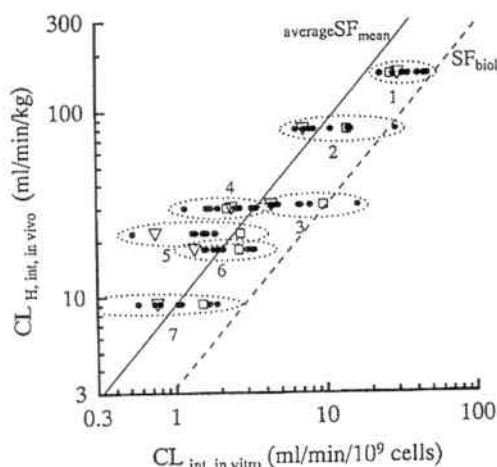


FIG. 1. $CL_{H, \text{int, in vivo}}$ versus $CL_{\text{int, in vitro}}$ in 10 cryopreserved preparations of human hepatocytes.

Each symbol represents the mean of three determinations. Data obtained from lot 70 and 73 are shown by open triangles and squares, respectively, whereas those in the individual preparations are shown by closed circles. Solid and broken line represent the predictions by the empirical scaling factor (average SF_{mean} in Table 1) and biologically based scaling factor (SF_{biol} , see Text), respectively, from 10 cryopreserved preparation.

among 10 cryopreserved preparations of human hepatocytes. The prediction of $CL_{H, \text{int, in vivo}}$ resulted in a marked underestimation when the biologically based scaling factor [3.1×10^9 cells/kg, calculated from the assumption that each gram of human liver contains 120×10^6 cells/g liver (Iwatsubo et al., 1997), and an average human has 1800 g of liver (Davies and Morris, 1993)] was used to extrapolate $CL_{\text{int, in vitro}}$ to $CL_{H, \text{int, in vivo}}$ (Fig. 1). Mean values for the empirical scaling factor (SF_{mean}) were calculated by averaging the ratio of $CL_{H, \text{int, in vivo}}$ to the corresponding in vitro values ($CL_{\text{int, in vitro}}$) for each standard compound (Table 1). The average SF_{mean} value among seven standard compounds was found to be 8.5×10^9 cells/kg, which was approximately 3 times larger than that of biologically based value (3.1×10^9 cells/kg). Reasonably accurate predictions were achieved (Table 1; Fig. 1) when the scaling factors, thus empirically obtained, were used for the extrapolation.

The averaged results from 10 or more preparations of human hepatocytes appeared to provide more reliable predictions for the human liver metabolism, whereas it was less convenient and cost-effective. It was found that the pooled preparation of two lots (lot 70 and 73) achieved the same extent of predictability for all seven standard compounds as the averaged results from 10 individual preparations (Tables 1 and 2). Pooled preparation demonstrated that the metabolic activity was constant during a 2-h incubation time period for standard compounds (Fig. 2). The in vitro- in vivo-correlation study was further extended to another seven compounds by using pooled cryopreserved preparation of human hepatocytes (Table 2). The predictions of $CL_{H, \text{predicted, 70+73}}$ and $F_{H, \text{predicted, 70+73}}$ were carried out with the average value of scaling factor (average SF_{70+73} , 10.8×10^9 cells/kg) for total 14 compounds, which was obtained empirically as described for 7 standard compounds in the pooled hepatocyte preparation of lot 70 and 73. Reasonably good correlations were obtained for both oral bioavailability (Fig. 3, panel A) and hepatic clearance (Fig. 3, panel B). These data demonstrated that the in vitro metabolic clearance obtained in the pooled preparation from cryopreserved human hepatocytes reasonably well predicted in vivo hepatic clearance and availability with the aid of empirical scaling factor.

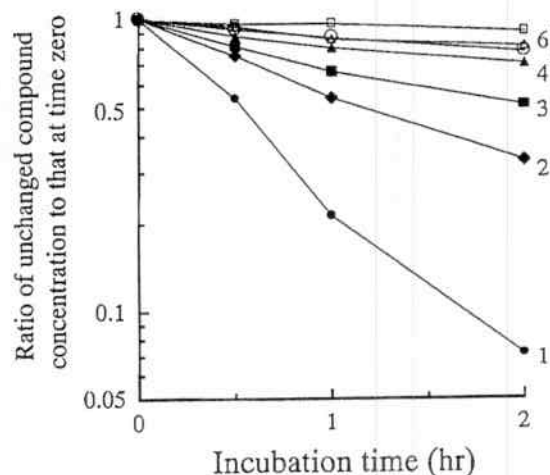


FIG. 2. Disappearance curves of seven standard compounds in the pooled human hepatocytes preparation (lot 70 and 73) suspended in serum.

Each symbol represents the mean of three determinations. The numbers correspond to the compound numbers listed in Table 1.

Discussion

Isolated hepatocytes have been recognized as more in vivo relevant in vitro systems than the subcellular fractions such as liver S-9 and microsomes for the prediction of in vivo metabolism. Although the freshly isolated human hepatocytes appeared to be one of the best preparations for the prediction of in vivo metabolism in humans (Lavé et al., 1999), the cryopreserved human hepatocytes instead became more prevalent and widely used for the routine analysis (Li, 2001). Cryopreserved human hepatocytes have been reported to quantitatively retain most of the phase I metabolic activities observed in the fresh liver, whereas some phase II metabolic activities to certain substrates were lower in the cryopreserved preparation than the intact human liver (Li et al., 1999; Steinberg et al., 1999; Hengstler et al., 2000; Rialland et al., 2000). In addition, consistent with the fact that each drug metabolizing enzyme activity in the human liver is known to individually vary between subjects, an approximately 3- to 5-fold variation was found in the in vitro metabolic clearance for the standard compounds among preparations from different human subjects (Table 1; Fig. 1). The interindividual variation in the metabolic capacity in the liver appears to reflect the observed large variation in the clearance in humans.

The empirical scaling factors for the in vitro-to-in vivo extrapolation (8.5×10^9 cells/kg from 10 individual preparations in Table 1; 10.8×10^9 cells/kg from pooled preparation in Table 2) were approximately 3 to 4 times larger than the anatomically calculated value (3.1×10^9 cells/kg). In addition, the variation of scaling factor obtained from 10 individual preparations (SF_{mean} in Table 1) between different compounds was much larger than that obtained from the pooled-cryopreserved preparation of human hepatocytes (SF_{70+73} in Table 2). These data suggested that the empirical scaling factor applicable only to the preparation used in the prediction was critically important for more reliable and rational predictions, which might compensate the inherent interindividual variation and/or loss of metabolic activities among different cryopreserved preparations.

In summary, the present study demonstrates that the direct evaluation of metabolic clearance in cryopreserved human hepatocytes in the presence of human serum was a convenient and useful tool for the prediction of hepatic clearance and availability. The calibration paradigm described in this report minimized the interindividual variation

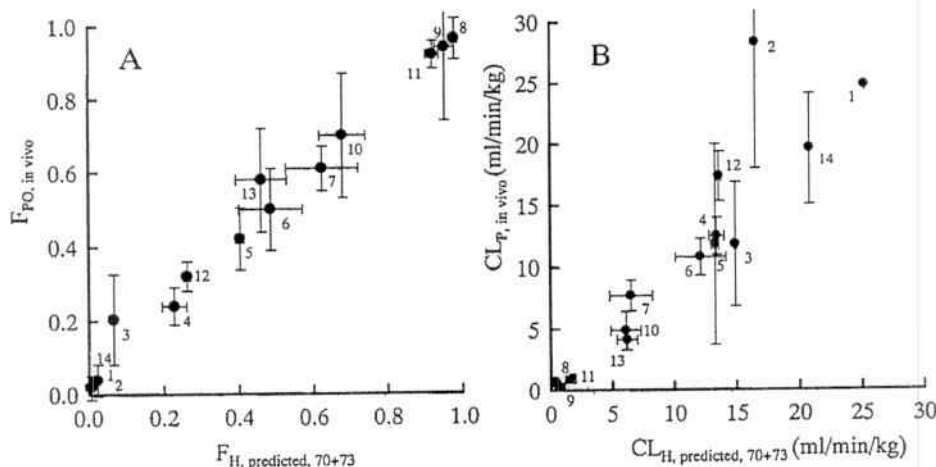


FIG. 3. Prediction of oral bioavailability (A) and hepatic clearance (B) using the cryopreserved preparation of human hepatocytes pooled from two separate lots.

The hepatocyte suspension was prepared by combining equal volumes of cryopreserved preparations of human hepatocytes from lot 70 and 73. Each symbol represents the mean \pm S.D. of three determinations of predicted values from cryopreserved preparations (x-axis) and those of observed values taken from the literature (y-axis). Numbers correspond to the compound numbers listed in Table 2. Predictions ($F_{PO, predicted, 70+73}$ and $CL_{H, predicted, 70+73}$) were carried out as described under the *Materials and Methods* section by using the average of empirical scaling factor (average SF_{70+73}) of 10.8×10^9 cells/kg (Table 2).

of metabolic activities among different subjects and improved the predictability of the in vitro data for the in vivo metabolic clearance with the aid of empirical scaling factor. The present method could be helpful at the early discovery stage to identify more promising candidates for further development that have lower hepatic clearance and higher oral bioavailability in humans.

References

- Asali LA and Brown KF (1984) Naloxone protein binding in adult and foetal plasma. *Eur J Clin Pharmacol* 27:459-463.
- Bertilsson L, Braithwaite R, Tybring G, Garle M, and Borga O (1979) Techniques for plasma protein binding of demethylchlorimipramine. *Clin Pharmacol Ther* 26:265-271.
- Blanchard J (1982) Protein binding of caffeine in young and elderly males. *J Pharm Sci* 71:1415-1418.
- Chan KK, Vyas KH, and Brandt KD (1987) In vitro protein binding of diclofenac sodium in plasma and synovial fluid. *J Pharm Sci* 76:105-108.
- Ciraulo DA, Barnhill JG, and Jaffe JH (1988) Clinical pharmacokinetics of imipramine and desipramine in alcoholics and normal volunteers. *Clin Pharmacol Ther* 43:509-518.
- Davies B and Morris T (1993) Physiological parameters in laboratory animals and humans. *Pharm Res* (NY) 10:1093-1095.
- Desoye G (1988) Error analysis in equilibrium dialysis: evaluation of adsorption phenomena. *J Biochem Biophys Methods* 17:3-16.
- Divoll M, Greenblatt DJ, Ochs HR, and Shader RI (1983) Absolute bioavailability of oral and intramuscular diazepam: effects of age and sex. *Anesth Analg* 62:1-8.
- Elstrom J and Lindgren S (1978) Influence of bed rest on the pharmacokinetics of phenazone. *Eur J Clin Pharmacol* 13:379-383.
- Gammans RE, Mayol RF, and LaBudde JA (1986) Metabolism and disposition of buspirone. *Am J Med* 80:41-51.
- Greenblatt DJ, Allen MD, Harmatz JS, and Shader RI (1980) Diazepam disposition determinants. *Clin Pharmacol Ther* 27:301-312.
- Gross AS, Heuer B, and Eichelbaum M (1988) Stereoselective protein binding of verapamil enantiomers. *Biochem Pharmacol* 37:4623-4627.
- Guentert TW, Holford NH, Coates PE, Upton RA, and Riegelman S (1979) Quinidine pharmacokinetics in man: choice of a disposition model and absolute bioavailability studies. *J Pharmacokinet Biopharm* 7:315-330.
- Hardy BG and Schentag JJ (1988) Lack of effect of cimetidine on the metabolism of quinidine: effect on renal clearance. *Inter J Clin Pharmacol Ther Toxicol* 26:388-391.
- Hengstler JG, Utesch D, Steinberg P, Platt KL, Diener B, Ringel M, Swales N, Fischer T, Biefang K, Gerl M, et al. (2000) Cryopreserved primary hepatocytes as a constantly available in vitro model for the evaluation of human and animal drug metabolism and enzyme induction. *Drug Metab Rev* 32:81-118.
- Holford N (1998) *Clinical Pharmacokinetics: Drug Data Handbook*, 3rd ed, ADIS Press Limited Auckland, New Zealand.
- Houston JB and Carlile DJ (1997) Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. *Drug Metab Rev* 29:891-922.
- Hughes IE, Ilett KF, and Jellett LB (1975) The distribution of quinidine in human blood. *Br J Clin Pharmacol* 2:521-525.
- Iwatsubo T, Hirota N, Ooie T, Suzuki H, Shimada N, Chiba K, Ishizaki T, Green CE, Tyson CA, and Sugiyama Y (1997) Prediction of in vivo drug metabolism in the human liver from in vitro metabolism data. *Pharmacol Ther* 73:147-171.
- Johansson KA, Appelgren C, Borg KO, and Elofsson R (1974) Binding of two adrenergic beta-receptor antagonists, alprenolol and H 93-26, to human serum proteins. *Acta Pharm Suec* 11:333-346.
- Lavé T, Coassolo P, and Reigner B (1999) Prediction of hepatic metabolic clearance based on interspecies allometric scaling techniques and in vitro-in vivo correlations. *Clin Pharmacokinet* 36:211-231.
- Li AP (2001) Screening for human ADME/Tox drug properties in drug discovery. *Drug Discov Today* 6:357-366.
- Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, and Silber PM (1999) Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability and drug-drug interaction potential. *Chem-Biol Interact* 121:17-35.
- Maguire KP, Burrows GD, Norman TR, and Scoggins BA (1980) Blood/plasma distribution ratios of psychotropic drugs. *Clin Chem* 26:1624-1625.
- McAllister RG Jr and Kirsten EB (1982) The pharmacology of verapamil. IV. Kinetic and dynamic effects after single intravenous and oral doses. *Clin Pharmacol Ther* 31:418-426.
- Nagy A and Johansson R (1975) Plasma levels of imipramine and desipramine in man after different routes of administration. *Naunyn-Schmiedeberg's Arch Pharmacol* 290:145-160.
- Newton R, Broughton LJ, Lind MJ, Morrison PJ, Rogers HJ, and Bradbrook ID (1981) Plasma and salivary pharmacokinetics of caffeine in man. *Eur J Clin Pharmacol* 21:45-52.
- Obach RS (1999) Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of in vitro half-life approach and nonspecific binding to microsomes. *Drug Metab Dispos* 27:1350-1359.
- Raafaub J and Dubach UC (1975) On the pharmacokinetics of phenacetin in man. *Eur J Clin Pharmacol* 8:261-265.
- Regardh CG, Borg KO, Johansson R, Johnsson G, and Palmer L (1974) Pharmacokinetic studies on the selective beta₁-receptor antagonist metoprolol in man. *J Pharmacokinet Biopharm* 2:347-364.
- Regardh CG, Jordo L, Ervik M, Lundborg P, Olsson R, and Ronn O (1981) Pharmacokinetics of metoprolol in patients with hepatic cirrhosis. *Clin Pharmacokinet* 6:375-388.
- Remmel RP, Copa AK, and Angaran DM (1991) The effects of hemodilution, pH and protamine on lidocaine plasma protein binding and red blood-cell uptake in vitro. *Pharm Res* 8:127-130.
- Rialland L, Guyomard C, Scotte M, Chesne C, and Guillouzo A (2000) Viability and drug metabolism capacity of alginate-entrapped hepatocytes after cryopreservation. *Cell Biol Toxicol* 16:105-116.
- Roberts MS and Rowland M (1986) Correlation between in-vitro microsomal enzyme activity and whole organ hepatic elimination kinetics: analysis with a dispersion model. *J Pharm Pharmacol* 38:177-181.
- Shibata Y, Takahashi H, and Ishii Y (2000) A convenient in vitro screening method for predicting in vivo drug metabolic clearance using isolated hepatocytes suspended in serum. *Drug Metab Dispos* 28:1518-1523.
- Steinberg P, Fischer T, Kiulies S, Biefang K, Platt KL, Oesch F, Bottiger T, Bulitta C, Kempf P, and Hengstler J (1999) Drug metabolizing capacity of cryopreserved human, rat and mouse liver parenchymal cells in suspension. *Drug Metab Dispos* 27:1415-1422.
- Vesell ES, Passananti GT, Glenwright PA, and Dvorchik BH (1975) Studies on the disposition of antipyrine, aminopyrine, and phenacetin using plasma, saliva and urine. *Clin Pharmacol Ther* 18:259-272.
- Walle T, Walle UK, Cowart TD, and Conradi EC (1989) Pathway-selective sex differences in the metabolic clearance of propranolol in human subjects. *Clin Pharmacol Ther* 46:257-263.
- Willis JV, Kendall MJ, and Jack DB (1980) A study of the effect of aspirin on the pharmacokinetics of oral and intravenous diclofenac sodium. *Eur J Clin Pharmacol* 18:415-418.
- Wilson TW, Firor WB, Johnson GE, Holmes GI, Tsianco MC, Huber PB, and Davies RO (1982) Timolol and propranolol: bioavailability, plasma concentrations and beta blockade. *Clin Pharmacol Ther* 32:676-685.
- Wing LM, Miners JO, Birkett DJ, Foenander T, Lillywhite K, and Wanwimolruk S (1984) Lidocaine disposition—sex differences and effects of cimetidine. *Clin Pharmacol Ther* 35: 695-701.

E.15

Cryopreserved Animal Hepatocytes

Non-human Primate						Metabolic activity pmol/10 ⁶ cells/min		
Species	Sex (# in pool)	Lot no.	Number of Vials	% Viability	ECOD	7-HCG	7-HCS	
Cynomolgus	M	Cy182(P)	1	91%	143	559	53	
Cynomolgus	M	Cy199	1	85%	374	701	57	
Cynomolgus	F	Cy216	71	83%	454	534	68	
Cynomolgus	M	Cy232	1	84%	248	859	86	
Cynomolgus	F	Cy235	78	91%	366	2940	282	
Cynomolgus	M	Cy241	12	90%	529	1680	32	
Cynomolgus	M	Cy257	18	86%	408	554	96	
Cynomolgus	M	Cy259	18	80%	100	563	98	
Cynomolgus	M	Cy265	77	92%	413	333	132	
Cynomolgus	M	Cy269	40	82%	197	410	81	
Cynomolgus	M (M6)	CyP10	77	82%	184	454	87	
Rhesus	M	Rh131	11	81%	151	297	12	

Rat

Rat						Metabolic activity pmol/10 ⁶ cells/min		
Species	Sex (# in pool)	Lot no.	Number of Vials	% Viability	ECOD	7-HCG	7-HCS	
Long Evans	F	Re100	119	82%	23	1456	465	
Long Evans	F	Rs437	76	90%	32	375	104	
Wistar	F	Rw123(P)	13	84%	30	394	105	
Sprague-Dawley	M	Rs430	1	84%	23	1975	463	
Sprague-Dawley	M	Rs439(P)	3	82%	22	594	140	
Sprague-Dawley	M	Rs472(P)	20	83%	29	1430	120	
Sprague-Dawley	M	Rs477	21	75%	31	1180	79	
Sprague-Dawley	M	Rs478(P)	98	87%	25	1640	460	
Sprague-Dawley	M	Rs494	23	82%	18	3480	700	
Sprague-Dawley	M	Rs504	15	83%	13	1560	517	
Sprague-Dawley	M	Rs509(P)	36	83%	40	1260	319	
Sprague-Dawley	M	Rs516	45	80%	179	616	312	
Sprague-Dawley	F	Rs513(P)	17	90%	25	1110	188	

Mouse

Mouse						Metabolic activity pmol/10 ⁶ cells/min		
Species	Sex (# in pool)	Lot no.	Number of Vials	% Viability	ECOD	7-HCG	7-HCS	
CD-1	M	Mc349(P)	18	91%	86	3940	63	
CD-1	M	Mc352	30	92%	117	5750	110	
CD-1	M	Mc355	5	81%	118	2390	77	
CD-1	M	Mc363	4	84%	294	2100	105	

Dog

Dog						Metabolic activity pmol/10 ⁶ cells/min		
Species	Sex (# in pool)	Lot no.	Number of Vials	% Viability	ECOD	7-HCG	7-HCS	
Beagle	M	Db128	395	79%	122	535	129	
Beagle	M	Db156	18	78%	139	462	254	
Beagle	M	Db176B	63	78%	291	407	117	
Beagle	M	Db180	4	88%	300	1430	257	
Beagle	M	Db192(P)	17	75%	447	676	99	
Beagle	M	Db193(P)	7	84%	253	1190	204	
Beagle	M	Db193(P)	39	84%	316	1360	200	
Beagle	M	Db193(P)	4	88%	239	728	129	
Beagle	M	Db195	34	88%	633	412	149	
Beagle	M	Db196a	43	93%	400	93	65	
Beagle	M	Db198	48	79%	389	288	57	
Beagle	M	DbP10	52	77%	413	263	102	

Rabbit

Rabbit						Metabolic activity pmol/10 ⁶ cells/min		
Species	Sex (# in pool)	Lot no.	Number of Vials	% Viability	ECOD	7-HCG	7-HCS	
New Zealand White	F	Ln108	3	88%	74	297	268	
New Zealand White	F	Ln113	44	71%	174	1760	137	
New Zealand White	F	Ln120	17	66%	360	274	68	
New Zealand White	F	Ln127(P)	43	64%	187	1373	172	
New Zealand White	M	Ln128(P)	27	87%	32	1996	128	

NOTES & ABBREVIATIONS:

TBD = data coming soon

VIABILITY denotes post-thaw results using the standard CellDirect protocol. This DOES NOT include the use of Percoll or

"specialized" media.

ECOD = 7-ethyl oxycoumarin-10-O-β-D-glucopyranoside (genetic Phase 1 metabolic test)

7-HCG = 7-hydroxycoumarin-7-glucuronide (Phase 1 metabolic test)

7-HCS = 7-hydroxycoumarin-7-sulfate (Phase 1 metabolic test)

(P) = pooled animal hepatocytes

p = pooled lot

Inventory Data Sheet

Metabolic assay conditions

General Phase I (CYP) and Phase II (UGT, SULT) drug metabolizing enzymes were analyzed for activity in each of the tests listed above. Cells were carefully thawed in Williams' E Medium (WEM) or Dulbecco's Modified Eagle Medium (DMEM) and resuspended to 1.0×10^6 cells/ml in incubation Medium (serum-free), 0.5 ml of cell suspension was aliquoted into a 12-well non-coated plate, respectively, containing the appropriate substrate and placed in a humidified incubator at 37°C, 95% relative humidity, and 5% CO₂ on an orbital

shaker. At incubations were performed in triplicate. At the appropriate time point for each substrate, a sample was collected from each well and stored frozen at -70°C until processed by LC/MS/MS or HPLC analysis. Metabolite formation was measured by standard biochemical assays using GC-MS/MS or HPLC analysis (Table 1). At least six calibration standards and 12 quality control samples (at three different concentrations) were used to evaluate the quality of analytical runs.

Table 1 substrate probes to assess human CYP3A50 metabolic activity.

Enzyme	Substrate	Concentration	Incubation time	Marker metabolite
Phase I	7-Ethoxycoumarin	100 µM	30 min	(ECOD) 7-Hydroxycoumarin (7-HC)
Phase II	7-Hydroxycoumarin	100 µM	30 min	7-Hydroxycoumarin Glucuronide (7-HCG) and 7-Hydroxycoumarin Sulfate (7-HCS)

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E.16

Cryopreserved Hepatocytes

Human Inventory

[Human Cryopreserved For Suspension Use - Metabolism Applications](#)
[Human Cryopreserved For Enzyme Induction Applications](#)
[Human Cryopreserved For Culture Use - In Biliary Excretion](#)
[Human Cryopreserved For Culture Use - In Plated Metabolism](#)

Animal Inventory

[Animal Cryopreserved Characterization/Inventory](#)

Example of a Product Characterization Sheet

[PCS-Lot# Hu0910](#)
[PCS-Lot# Hu0939](#)
[PCS-Lot# Hu8064](#)
[PCS-Lot# Hu8072](#)
[PCS-Lot# HuP58](#)

CellzDirect has built an extensive inventory of superior grade animal and human cryopreserved hepatocytes. We have carefully produced and characterized our lots, offering detailed donor demographics, extensive P450 profiling, responsiveness to inducers, morphological assessment, and application suitability. We offer pooled and single donor human and animal lots, including an ample supply of plateable lots from all standard toxicological species that are suitable for a variety of applications. For your convenience we offer application-specific kits for thawing and plating. Please review our current animal and human inventory lists and characterization data. Experienced scientists in our Technical Support & Customer Service Department are standing by to assist you:

1-866-952-3559 or +1-919-545-9959 hepaticproducts@invitrogen.com

Cryopreserved hepatocytes have more recently become mainstays for *in vitro* researchers worldwide. Responding to this demand, CellzDirect scientists, partnered with APS, Inc., have worked painstakingly to develop a large inventory of high quality human cryopreserved cells. We offer five basic application-based categories of cryopreserved hepatocytes: Hepatocytes For Suspension Use - Metabolism Applications, Pooled Human Cryopreserved For Suspension Use - Metabolism Applications, Hepatocytes For Culture Use - Metabolism Applications, Hepatocytes For Enzyme Induction Applications and Human Cryopreserved For Biliary Excretion Applications. For your convenience, we offer a Cryopreserved Hepatocyte Evaluation Kit (CHEK) and application medium.

Cryopreserved Hepatocyte Standard Species Available

Human, Non-human primate (Baboon, Cynomolgus, Rhesus), Dog (Beagle), Rabbit (New Zealand White), Rat (Sprague-Dawley, Wistar), Mouse (CD-1)

Custom Species Available Upon [Request](#)

Lot Characterization

Due to the wide variety of applications cryopreserved hepatocytes are used for, CellzDirect has worked to provide the most thorough lot characterization data in the industry. Extensive characterization information is provided with each lot that you purchase and minimally includes:

- *Post Thaw Cell Viability and Yield
- *Incubation Stability
- *Phase I & II Enzyme Activities
- *Morphological Assessment with Representative Images

Our **human plateable** lots undergo additional evaluation which includes a suitability assessment for common application such as: metabolic stability, enzyme induction, and biliary excretion. To provide you with the best plating conditions for your specific application, we offer optimized plating and culture conditions. As part of this evaluation, we include a multi-day quality and functional assessment of the monolayer and complete characterization of the induction profile for each lot.

- *Donor Demographics
- *Optimized Seeding Density
- *Attachment Efficiency
- *Morphological Assessment of the Monolayer with Representative Images
- *Suitability for Drug Transport Studies
- *Cytochrome P450 Induction (CYP1A2, CYP2B6, and CYP3A4)

Formats and Shipping

CellzDirect's cryopreserved hepatocytes are from male animals unless otherwise requested. The human hepatocytes are from both

male and female, depending on availability. Cryopreserved hepatocytes are shipped Monday through Thursday by FedEx Priority. Orders received after 12 P.M. EST are shipped the following business day. Contact us if you require special shipping arrangements.

For research purposes only; not to be used for clinical application or redistribution. CellzDirect cell products may contain human or other primate source material that should be treated as potentially hazardous. Use universal precautions for handling biohazardous material.